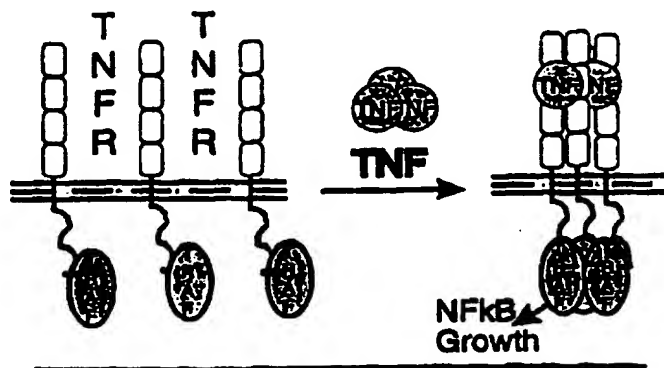




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(54) Title: CONTROLLING TRAF-MEDIATED SIGNALS



## (57) Abstract

Compounds and methods for interrupting the interaction between Epstein-Barr virus encoded proteins known as LMP1 and Tumor Necrosis Factor Receptor Associated Factors (TRAFs), particularly novel human TRAFs, thereby inhibiting lymphoblast growth and tumorigenesis, particularly Hodgkin's disease, Burkitt's lymphoma, lymphomas seen in immunocompromised patients (including AIDS-associated central nervous system lymphomas), and nasopharyngeal carcinomas. Therapies for treating EBV infection are also disclosed, e.g. in patients with infectious mononucleosis, by blocking the establishment of latent infection and/or blocking lytic infection. Compounds and methods for controlling TRAF-Mediated TNF/TNFR signaling by administering to a TRAF-encoding cell a compound that inhibits TRAF oligomerization are also disclosed. Compounds and methods for controlling cell growth and death based on the interaction of TNF receptor family carboxy terminal cytoplasmic domains with human TRAFs, e.g., LAP1 and EBI6. These interactions are particularly important in controlling cells in the immune system and regulating immune responses. They are also important for controlling abnormally growing cells, that is cancer cells.

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## CONTROLLING TRAF-MEDIATED SIGNALS

### Field of the Invention

5           This invention is in the general field of compounds (including proteins) and methods for controlling cell growth (or tumorigenesis), particularly cell growth/tumorigenesis related to viral infections, such as Epstein-Barr virus (EBV) infection. The  
10 invention also relates to therapeutic strategies for treating viral infections and conditions characterized by abnormal or undesired immune functions.

### Background of the Invention

#### I. Epstein-Barr Virus LMP1

15           EBV is a herpesvirus that infects B lymphocytes and certain epithelial cells. EBV causes lymphoproliferative diseases and tumorigenicity in humans, which manifest particularly as infectious mononucleosis, Hodgkin's disease, Burkitt's lymphoma,  
20 lymphomas seen in immunocompromised patients (including AIDS-associated central nervous system lymphomas), and nasopharyngeal carcinomas, the latter being particularly prevalent in populations of southern Chinese extraction. See, Kieff and Liebowitz, "Epstein-Barr virus and its  
25 replication" *Virology*, B.N. Fields and D.M. Knipe, eds., New York Raven Press, pp. 1889-1920 (1990); and Miller, "Epstein-Barr Virus" *Virology*, B.N. Fields and D.M. Knipe, eds., New York Raven Press, pp. 1921-1958 (1990).

          An EBV-encoded protein termed latent infection  
30 membrane protein 1 (LMP1) induces most of the phenotypic effects of EBV B-lymphocyte infection. LMP1 has been characterized as an integral membrane protein which consists of a 23 amino acid amino terminal cytoplasmic domain, six markedly hydrophobic transmembrane domains  
35 separated by short reverse turns, and a 200 amino acid

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carboxy terminal cytoplasmic domain (Fennewald, et al., *J. Virol.* 51:411-419 (1984); and Hennessy, et al., *Proc. Natl. Acad. Sci. USA* 81:7201-7211 (1984)). The transmembrane domains enable LMP1 to post translationally  
5 insert into membranes and to accumulate in aggregates in the plasma membrane (Hennessy, et al., *Proc. Natl. Acad. Sci. USA* 81:7201-7211 (1984); Liebowitz, et al., *J. Virol.* 58:233-237 (1986).

LMP1 has been implicated as essential for  
10 B-lymphocyte transformation (Kaye et al., *Proc. Natl. Acad. Sci. USA* 90:9150-9154 (1993)). From the observation that LMP1 forms patches in the plasma membrane, Wang et al., (1985) *Cell*, 43:831-840 suggest that it could be part of a complex that might play a  
15 direct role in virus induction of cell proliferation. Based on LMP1's capacity to transform established cells, Wang et al. conclude that LMP1 mimics the cell growth effect of tyrosine kinase oncogenes and ras genes. In so doing, Wang et al. point out that LMP1 is not  
20 significantly homologous to any of those oncogene products, and they conclude that it may affect cell growth by a different mechanism. They further conclude that LMP1 is unlikely to be a receptor for a growth factor and, by analogy to other oncogenic events which  
25 frequently involve more than one gene, they conclude that EBV nuclear protein genes are likely to be necessary complements to LM1P in the growth transformation of primary B cells. Finally, they speculate that patching could be important in the biologic effect of LMP1,  
30 because: a) LMP could interact with a growth factor receptor; and b) growth factors are essential to the continued proliferation of EBV transformed lymphocytes. Kaye et al., *Proc. Natl. Acad. Sci. USA* 90:9150-9154 (1993) also indicate that LMP1 transmembrane domains are  
35 important for conferring plasma membrane aggregation.

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## II. TNF/TNFR Signaling

Tumor necrosis factor (TNF) is a cytokine produced mainly by activated macrophages, which elicits a wide range of biological effects related to endotoxic shock, inflammatory, immunoregulatory, proliferative, cytotoxic, and anti-viral activities. See generally Tartaglia and Goeddel (1992) *Immunology Today* 13:151-153; Goeddel et al. (1986) *Cold Spring Harbor Symp. Quant. Biol.* 51:597-609; Beutler and Cerami (1988) *Ann. Rev. Biochem.* 57:505-518; and Fiers (1991) *FEBS Lett.* 285:199-212. The various cellular responses mediated by TNF are initiated by its interaction with two distinct cell surface receptors of approximately 55kDa (TNF-R1) and 75kDa (TNF-R2). See Tartaglia and Goeddel, cited above; and Rothe et al. (1992) *Immunol. Res.* 11:81-90. These receptors are also known, respectively as p60TNFR and p80TNFR. The independent signaling responses mediated by these two receptors have been studied. See Rothe et al. (1994) *Cell* 78:681-692 and publications cited therein. Both receptors are members of the larger TNF receptor superfamily having certain common structural and functional characteristics. Other TNFR family members include CD30, CD27, CD40, lymphotoxin- $\beta$  receptor, OX-40, 4-1BB, and CD95 (Fas). See generally Smith et al. (1994) *Cell*, 76:959-962; and Beutler and van Huffel (1994) *Science*, 264:667-668.

TNF-receptor superfamily signaling (including NF $\kappa$ B signaling) is involved in cell growth and cell death. Such signaling appears to require the aggregation of receptor monomers in a process initiated by ligand binding. The multimerization of receptor cytoplasmic domains is postulated to unveil discrete chain motifs or to create composite tertiary epitopes that attract or activate constitutively associated molecules that are components of intracellular signal transduction pathways.

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See, Bazan (1993); and Tartaglia et al. (1993b). In general, notwithstanding attempts to implicate second messenger systems in TNF signaling, the mechanism for coupling of second messenger systems to TNF receptors remains an enigma. See, Pfizenmaier et al. (1992); Kolesnick and Golde (1994); and other articles cited in Rothe et al. (1994), cited above.

Rothe et al. (1994) report cloning of two murine signal transducers associated with the cytoplasmic domain of a member of the TNF receptor superfamily (TNF-R2). They designate those transducers TRAF1 and TRAF2. They report that TRAF1 and TRAF2 form a heterodimeric complex associated with the cytoplasmic domain of murine TNF-R2 (see Figure 8 of Rothe et al.). They conclude:

"By virtue of their ability to associate with the cytoplasmic domain of TNF-R2, TRAF1 and TRAF2 define a novel class of putative signal transducers. However, their functional roles and mechanisms of action in TNF-R2-mediated signaling are presently unresolved. Of particular interest will also be their mode of action after binding of the ligand TNF to the receptor."

#### Summary of the Invention

There are several aspects of the invention separately summarized below.

##### **I. Inhibiting LMP1-LAP1 Interaction**

We have discovered a novel B-cell protein which we term "LMP1 Associated Protein 1 or LAP1", which strongly associates with a cytoplasmic carboxy terminal domain of EBV LMP1. This LMP1 domain is an essential component of LMP1 for effecting cell growth transformation. LAP1 is related to murine TRAF2. We have also discovered a related novel B cell protein induced by EBV infection, which we term Epstein-Barr Induced protein 6 or EBI6.

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EBI6 has extensive homology to murine TRAF1 and appears to be its human homolog.

Our findings are that: a) LMP1 expression clusters LAP1 and EBI6 to lymphoblast plasma membrane patches; b) LMP1 co-immunoprecipitates with LAP1 or EBI6; c) LAP1 can directly and biochemically interact with LMP1, particularly with a 44 amino acid segment of the LMP1 carboxy terminal cytoplasmic domain that is stringently required for transformed cell growth; d) LAP1 directly and biochemically interacts with the cytoplasmic domains of p80TNFR, CD40 and the lymphotoxin- $\beta$  receptor; e) to a lesser extent, LAP1 interacts with the cytoplasmic domains of p60TNFR and Fas; f) EBI6 associates strongly with p80 TNFR and, to a lesser extent, with p60TNFR.

We conclude that the lymphoblast growth and tumorigenesis effects of EBV depend on LMP1-associated TNFR-signal transduction, and that the TRAFs represent a necessary link in such EBV-induced signal transduction. Specifically, we conclude that TRAF oligomerization effected by LMP1 interaction is a necessary step in EBV-induced lymphoblast growth and tumorigenesis. See Figure 6B.

A particularly functional use for our discovery involves new screening techniques, compounds and methods for interrupting the LMP1-related, TRAF-mediated signaling and thus inhibiting lymphoblast growth and tumorigenesis. This use of the invention applies generally to virally induced tumors (particularly but not exclusively of lymphoid cells). The use particularly applies to Hodgkin's disease, Burkitt's lymphoma, lymphomas seen in immunocompromised patients (including AIDS-associated central nervous system lymphomas), and nasopharyngeal carcinomas. The invention also provides therapies for treating viral infection, e.g. in EBV-infected patients with infectious mononucleosis, by

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blocking the establishment of latent infection and/or blocking lytic infection. While we postulate a specific molecular mechanism, the effectiveness of the therapeutic compounds and methods described herein and the practice  
 5 of the invention do not depend on the completeness or accuracy of (and we do not wish to bind ourselves to) any particular theory.

Accordingly, one aspect of the invention features methods of treating infection and controlling cell  
 10 growth/tumorigenesis associated with a LMP1-encoding viruses (particularly EBV), by administering to infected cells a compound that inhibits TRAF-mediated virally induced TNFR cell growth, cell death, and/or NFκB signaling. It particularly features inhibiting  
 15 interaction between EBV LMP1 and a Tumor necrosis factor Receptor Associated Factor (TRAF).

Two categories of compounds that inhibit LMP1-TRAF interaction are: A) polypeptides that interact with LMP1, particularly with a 44-amino acid TRAF-interacting domain  
 20 of LMP1 (amino acids 188-231) discussed below;<sup>1</sup> and B) polypeptides that interact with TRAF proteins at a LMP1-interacting TRAF domain, at a TRAF-TRAF interacting domain that is necessary for TRAF oligomerization (or TRAF-TRAF interaction), or at a TNFR-interacting TRAF  
 25 domain.

#### A. LMP1-1 Interacting Inhibitors

Examples of category A above (LMP1-interacting inhibitors) are polypeptides that include a LMP1-interacting TRAF domain, particularly a LMP1-interacting  
 30 domain of a human TRAF such as a LAP (most preferably LAP1). The TRAF domain of the inhibitors may be a LMP1-

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<sup>1</sup> The sequence (SEQ ID NO: 3) is a 44 amino acid sequence at the beginning of the C-terminal cytoplasmic domain; see Fennewald et al. *J. Virol.*, 51:411-419 (1984),  
 3 Hereby incorporated by reference: G Q R H S D E H H H D D S L  
 P H P Q Q A T D D S G H E S D S N S N E G R H H L L V S G A.



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binding TRAF epitope contained on the C-terminal side of the TRAF coiled coil (Leu zipper) domain described below, for example an epitope on the LAP1 segment represented by the hybrid protein described below, G4TADLAP1 (amino acids 346-568 of SEQ ID NO:1). Those skilled in the art will readily recognize that specific LMP1-interacting LAP1 sequences can be identified using standard techniques by making proteolytic (e.g., V8 enzyme or trypsin) or recombinantly produced fragments of the above LAP1 segment (aa 346-568). The resulting fragments are then tested for interaction using any of the screens described elsewhere in this application. One LAP1 sequence of interest includes amino acids 346-368 of SEQ ID NO:1 -- E A D S M K S S V E S L Q N R V T E L E S V D. A second LAP1 sequence of interest is the C-terminal sequence (amino acids 407-568 of SEQ ID NO:1). If the LMP1-interacting domain is not contained therein, these sequences can be lengthened in an iterative process or other fragments may be screened until a minimal LMP1-interacting LAP1 sequence is identified.

#### B. TRAF-protein-interacting Inhibitors

One class of category B (TRAF-interacting) inhibitors are polypeptides that include a LMP1 sequence (particularly the above-described 44-amino acid sequence, SEQ ID NO: 3)) that interacts with a TRAF protein such as a human LAP, particularly human LAP1, and can therefore prevent the TRAF protein from interacting with LMP1. A second class of TRAF-interacting inhibitors are polypeptides that include a TRAF (e.g., a human TRAF such as EBI6 or a human LAP such as LAP1) oligomer-forming coiled coil domain -- i.e., a TRAF domain involved in formation of TRAF-TRAF hetero- or homo-oligomers. Such domains can be recognized by a repeating pattern in which every seventh (more or less) amino acid residue is Leu,

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Ile, or Val. See generally, Figure 1. One specific such domain includes the LAP1 sequence from amino acids 309-341 of SEQ ID NO:1, inclusive: L R N N E S K I L H L Q R V I D S Q A E K L K E L D K E  
5 I R P F R. A second specific such domain includes the EBI6 sequence from amino acids 194-224 of SEQ ID NO:2, inclusive: L R V F E N I V A V L N K E V E A S H L A L A T S I H Q S Q L.  
Either of the above domains can be extended, for example,  
10 to include the LAP1 sequence between amino acids 263-400 or EBI6 amino acids 187-257. As described above with respect to LMP1-LAP1 interaction, oligomerizing (or TRAF-interacting) LAP1 or EBI6 fragments can be identified using standard fragmentation and screening techniques, in  
15 which the above fragments, or longer polypeptides containing all or a significant part of them, are tested for TRAF interaction.

A third class TRAF interacting inhibitors includes inhibitors that have a TNFR (e.g., TNFR p60 or p80) TRAF-  
20 interacting domain. This third class of inhibitors is used primarily in the second aspect of the invention (II., below), and so we will describe it later with respect to that aspect of the invention. Nevertheless, it is possible that these inhibitors will also inhibit  
25 LMP1-TRAF interaction.

This first aspect of the invention specifically includes the polypeptide inhibitors described in the above methods and recombinant DNA encoding those inhibitors. Preferably, the recombinant nucleic acid  
30 further comprises regulatory DNA positioned to transcribe the polypeptide encoding DNA. The recombinant DNA itself may be formulated for use in gene therapy, --i.e., for administration to the patient as DNA expressed in the patient to produce the therapeutic polypeptide, or it may  
35 be used as a reagent to produce therapeutic polypeptides

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in conventional fermentation processes. In either case, the invention may also feature cells comprising the above recombinant nucleic acid, including the regulatory DNA required to express the polypeptide. The invention also  
5 includes methods of making the purified polypeptide by culturing a cell comprising the recombinant nucleic acid and recovering the purified polypeptide from the cells or the culture medium. The method also includes recombinant polypeptides and antibodies thereto -- i.e.,  
10 antibodies raised against, or antibodies that specifically bind to, the polypeptides.

This aspect of the invention is particularly useful for treating EBV-infected patients described above. Details of the invention will be apparent from  
15 the description below of TRAFs and of methods that establish and evaluate inhibition of the LMP1-TRAF interaction.

This aspect of the invention also features one of the above-described TRAF signal-effecting inhibitors  
20 formulated for administration as a therapeutic. Further, it features polypeptides that have been modified as described in greater detail below, while preserving the inhibitory effect of the native sequence.

This aspect of the invention also features  
25 procedures for screening candidate compounds for their ability to inhibit LMP1-TRAF or TRAF-TRAF interaction. *In vitro* and *in vivo* screens are described in greater detail below.

The peptides of the invention may also be used as  
30 specialty chemicals to control growth of EBV-transformed cell cultures.

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## II. Controlling TRAF-Mediated TNF/TNFR Family Cell Growth/Death Signaling

A second major aspect of the invention features controlling TRAF-mediated cell growth/death signaling via the TNFR superfamily of receptors. As described above, this family includes a number of receptors characterized by common functional and structural features. For a general review of TNFR's, see Smith et al., *Cell* 76:959-962, (1994) hereby incorporated by reference). The invention includes controlling TNFR cell growth/death signals that are a response to direct interaction with the receptor ligand, or (as described above) a response to interaction with other macromolecular species such as LMP1. It also includes controlling signals that result from mutations in receptors that lead to abnormal cell growth/death signaling. We specifically include (without limitation) signaling via TNFR family members such as p80, CD40, and lymphotoxin- $\beta$  receptor that interact directly with LAP1. Less preferred instances of TNFR signaling controlled by the invention include p60 and Fas signaling.

Specifically, our discovery indicates that TRAFs are an important component in TNF-TNFR cell growth/cell death or NF $\kappa$ B signaling. Indeed, LAP1 interacts directly with p80, LT $\beta$ , CD40, and (to a lesser extent) with p60 and Fas, and such interaction is an essential step in the signal pathway. Thus, the second aspect of the invention features controlling TRAF-mediated TNFR cell growth/death signals independent of EBV or other viral infection.

In this aspect of the invention TRAF-Mediated TNF/TNFR signaling is controlled by administering to a TRAF-encoding cell a compound that interacts with a TRAF or with a TNFR to inhibit signaling function.

Preferred compounds are those that interact with a TRAF, for example, with an oligomerizing TRAF domain,

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particularly an oligomerizing domain of a human TRAF. Inhibitors according to this aspect of the invention include polypeptides having a coiled coil TRAF domain. One such human TRAF domain is a human EBI6 coiled coil domain, for example, the EBI6 sequences described above. Human LAP (particularly LAP1) coiled coil domains described above are also included. Inhibitors containing the coiled coil domain of LAP1 or (more preferably) EBI6 described below will competitively bind to TRAFs without effecting a signal because such binding will inhibit TRAF oligomerization and thereby inhibit the TNF-TNFR growth signal pathway.

A second class of TRAF-mediated signaling inhibitors are those that directly involve other TRAF domains, such as the C-terminal TNFR-interacting LAP1 domain contained in amino acids 407-568 of LAP1 (SEQ ID NO:1) or the EBI6 domain contained in amino acids 259-416 of EBI6 (SEQ ID NO:2). The metal binding domains (see the RING finger motif of LAP1 and the zinc finger structure of EBI6 that are underlined in Figure 1) are likely to chelate to metal ions and function as mediators of macromolecular interactions in the TNFR cell growth/death signaling. Thus, these metal binding domains are also candidate inhibitors of such signaling.

Administration of these inhibitors is indicated for patients with undesired lymphocyte proliferation, particularly autoimmune diseases such as rheumatoid arthritis, Crone's disease, lupus, or patients requiring immunosuppression for organ transplantation.

As above, this aspect of the invention includes not only polypeptide inhibitors and effectors, but also the DNA encoding them and cells containing that DNA, for use as a therapeutic or as biological materials or as reagents in a method for making them. It also includes recombinant polypeptides and antibodies to the

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polypeptides. Similarly, this aspect of the invention features *in vivo* and *in vitro* screens for identifying compounds that inhibit TRAF oligomerization and that mimic the TRAF TNF-TNFR signal-mediation function.

5           This aspect of the invention particularly includes treatments of patients with non-EBV-associated Hodgkin's disease. It is interesting to note that high CD30 level found in Hodgkin's disease may result from faulty TRAF, acting as an oncogene to disrupt the TNFR family-mediated  
10 death signals or their cell growth effects. This aspect of the invention also includes treatment and diagnosis of patients characterized by a TRAF mutation.

Enhanced cell control of the cell death signal might be desirable in treatment of other tumors. For  
15 example, some viruses may disrupt the immune response by disrupting the normal TNFR cell growth/death signal (e.g., by secreting ineffectual TNF-competitors that prevent TNFR activation). Others may disrupt the cell death signal by acting downstream at a TRAF-mediated  
20 portion of the signal pathway. In either event, such viruses may be treated by the addition of TRAFs or TRAF domains that are effective to mediate the cell death signal, notwithstanding the viral intervention.

Alternatively, compounds identified in the screens below  
25 as augmenting the cell death signal could be used.

Thus, this aspect of the invention may have application to virally induced neoplasms that are not characterized by a LMP1-type molecule, such as hepatitis virus-induced hepatocarcinoma, human papilloma virus-  
30 induced cervical cancer, and adult T-cell leukemia induced by HTLV-I.

Finally, control of TRAF-mediated cell growth/death may be useful as a generalized transient immune enhancer (adjuvant) to accompany a specific

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vaccine for inducing immunity to infectious agents or abnormal cells.

This aspect of the invention also features enhancing TNF/TNFR cell growth/death signals by providing additional TRAF to mediate those signals. We have discovered two specific proteins useful in such signal enhancement, which are Human LMP1-Associated Protein (LAP1) and Human Epstein-Barr virus Induced Protein-6 (EBI6).

10 Other embodiments are within the following claims.

Description of the Preferred Embodiments

As summarized above, one aspect of the invention features inhibiting interaction between a viral LMP1 and a TRAF. Figure 6B is a diagram of this interaction, which ultimately results in NFkB mediated growth signaling. At the outset, we emphasize that our invention extends beyond the narrow bounds of the experiments described in detail below. It specifically extends beyond EBV and LAP1.

20 A second aspect of the invention focuses on TRAF-mediated signaling that does not require LMP1, as illustrated by Figure 6A. In this aspect, such signaling is inhibited as described above.

*I. The Viruses Being Inhibited*

25 While EBV is the only herpesvirus currently known to encode a LMP1 protein and our discussion of this aspect of the invention concentrates on treatment of EBV-infected patients, our discoveries concerning the mechanism of EBV-induced cell growth and tumorigenesis can be readily applied to other viruses that induce cell growth and/or tumors or that block cell death through a TNFR family member-interacting TRAF. The target viruses also preferably (but not necessarily) will encode a membrane protein having comparable structure and

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function; alternatively, the virus may encode a cytoplasmic protein that aggregates to activate TRAFs.

Structurally, LMP1 proteins are viral proteins that include N- and C-terminal cytoplasmic domains, 5 connected by multiple intervening hydrophobic transmembrane domains separated by short reverse turns. These transmembrane domains enable the protein to post translationally insert into membranes and to accumulate in aggregates (oligomers) in the plasma membrane. 10 Functionally, LMP1s have broad transforming effects as membrane aggregated proteins. Presumably they activate a growth factor receptor pathway.

Accordingly, our invention broadly features inhibiting interaction with LMP1 proteins, and we do not 15 limit ourselves in this aspect of the invention to EBV therapies. Preferably, the invention features EBV-related therapies, including therapies for infection with any EBV strain or type.

## *II. Characteristics Of TRAF Mediators Being Inhibited*

20 As discussed above, our discovery relates to specific signaling pathways mediated by TRAFs. We use the term TRAF to describe the family of signal transducing proteins that mediate TNF/TNFR cell growth/death and NFkB signals. TRAFs are characterized 25 by association with the cytoplasmic domain of a Tumor Necrosis Factor Receptor. The TRAFs of interest in this aspect of the invention can be identified by the following characteristics.

First, TRAFs include a C-terminal intracellular 30 domain that begins with an extended coiled coil motif. The coil motifs of different TRAF molecules could be involved in oligomerization of these molecules and generation of complexes involved in signaling. Some, but not all, TRAFs interact strongly with LMP1 proteins 35 (described above). For example, they interact with the



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above-described 44 amino acid LMP1 domain implicated in B cell growth and tumorigenesis. TRAFs are generally capable of interacting with the cytoplasmic domains of multiple TNFR's as described above (e.g., the 78 C-terminal amino acids of TNF-R2). It is possible that the same TRAF domain that interacts with LMP1 also interacts with the cytoplasmic domain of a TNFR. Some, but not all, TRAFs contain an N-terminal RING finger sequence motif -- a cysteine rich protein motif related to other zinc finger motifs involved in protein-DNA and protein-RNA interaction. Methods and procedures for identifying further TRAFs will be apparent from the following description of our discovery of LAP1 and EBI6 and from the above description of screening methods.

We have specifically identified two human TRAFs and confirmed their direct biochemical association with the various members of the TNF receptor family. Thus, LAP1 interacts directly with the cytoplasmic domain of TNFR p80, LT $\beta$ , CD40, and (to a lesser extent) with p60 and Fas. A second novel human TRAF, EBI6 interacts directly with the p80 cytoplasmic domain and (to a lesser extent) with the p60 cytoplasmic domain. Also, in the case of LAP1, we also have confirmed interaction with LMP1.

The TRAF domain may be defined on the basis of maximal (>50%) collinear primary sequence identity with the carboxy terminal 230 amino acids of a known TRAF, such as the murine TNF receptor associated proteins TRAF1 and 2. Genetic and biochemical data link TRAF1 and 2 to the cell growth, cell death, and NF- $\kappa$ B transducing effects of a domain near the carboxy terminus of the p80 TNF receptor cytoplasmic tail (Rothe, et al., *Cell* 78:681-692 (1994)).

The sequence of LAP1 and EBI6 is compared to TRAF1 and 2 in Figure 1. Specifically, Figure 1 depicts

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alignment of the amino acid sequences of human EBI6 and LAP1 and murine TRAF1 and TRAF2 using the CLUSTAL program (PCGene; IntelliGenetics). Identical (stars) and homologous (dots) amino acids are shown. Pairwise alignment of EBI6 and TRAF1 is also shown with identical amino acids designated by bold faced characters. Amino acids that form the RING finger motif in LAP1 and TRAF2 and the Zn finger structure in EBI6 and TRAF1 are underlined. Amino acids that form putative coiled coil structures are boxed. The TRAF domain is shown by large boxes.

EBI6 is almost certainly the human homolog of murine TRAF1 based on collinear 86% primary sequence identity, the expression of both in lung but not in most other tissues, and the amino terminal zinc finger motif. LAP1 is not the human homolog of murine TRAF2, but rather the existence of these two molecules is indicative of a larger repertoire of ring finger TRAFs. LAP1 is similar to TRAF 2 in size, in having an amino terminal RING finger domain, and in being constitutively expressed in most tissues. However, while LAP1 is 45% identical to both TRAF 2 and TRAF 1 in the TRAF domain, LAP1 is quite divergent from TRAF2 outside of the TRAF domain and is only 27% identical to TRAF 2 overall. LAP1 also appears to differ from TRAF2 in not interacting with EBI6, the human homolog of TRAF1.

As demonstrated by the examples below, LAP1 and EBI6 interact with the cytoplasmic domain of not only the p80 TNF receptor but also p60, albeit less strongly than with p80. This is the first evidence of interaction beyond p80 between these putative effectors and the TNF receptor family. Indeed, LAP1 can also interact strongly with the CD40 cytoplasmic domain as well as the lymphotoxin- $\beta$  receptor, and, to a lesser extent, with Fas cytoplasmic domain (Figure 5B).

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We have identified human TRAFs in the context of an investigation into the mechanisms by which LMP1 transforms cells, and a significant result of these experiments is the establishment of a connection between LMP1 and TNF receptor signaling pathways. LMP1 binds directly to LAP1 and also interacts with EBI6 in human lymphoblasts. Our genetic, biochemical and intracellular localization data on the interaction of LMP1 with LAP1 and EBI6 reinforce the previous genetic and biochemical linkage of murine TRAF 1 and 2 to the TNF receptor cytoplasmic domains in supporting a role for the TRAFs as mediators of growth/death and NF- $\kappa$ B responses.

Figures 6A and 6B are schematic models of TRAF mediated signal transduction. Figure 6A shows p80 TNF receptor activation as described above and Figure 6B shows LMP1 complexes (Figure 6B) at the plasma membrane. A model for the activation of the p80 TNF receptor is shown in Figure 6A. The extracellular region of the TNF receptor is composed of four domains with characteristic cysteine patterns. The cytoplasmic domain of the receptor is known to associate with TRAF molecules (TRAF). Upon binding of TNF (shown here as a trimer) the extracellular domains of several receptor molecules are believed to be crosslinked causing aggregation of intracellular domains and their associated TRAF molecules. Clustering of receptor molecules and their intracellular domains results in signal transduction as manifested by a number of phenotypic alterations including the activation of the transcription factor NF- $\kappa$ B and cell growth or death signaling.

In Figure 6B three LMP1 molecules are shown to form a constitutive complex at the plasma membrane (depicted by the gray area between the two solid horizontal lines). The amino terminal (N) and carboxy terminal (C) cytoplasmic regions of LMP1 are shown by

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short and long lines respectively. The transmembrane domains of LMP1 are depicted by vertical cylinders which are joined by short reverse turns (short curved lines). Aggregation of LMP1 molecules at the plasma membrane  
5 brings together LMP1 associated TRAF molecules (TRAF) in a complex thus generating a constitutive signal that results in pleiotropic effects including activation of NF- $\kappa$ B cell growth signaling.

Additional TRAF proteins are likely to emerge  
10 with screening, generally as described below in Example I for LAP1 and EBI6. LAP1 was identified twice in a our yeast two hybrid screen of  $5 \times 10^5$  cDNAs and no other strong interaction was identified, providing a direct and possibly exclusive linkage between LMP1 and LAP1. In  
15 yeast, the interaction of LAP1 with LMP1 is through the membrane proximal 44 amino acids of LMP1 and the last 223 residues that comprise the LAP1 TRAF domain. The interaction with the first 44 amino acids of the LMP1 carboxy terminus is significant since EBV recombinants  
20 deleted for this region apparently do not initiate fibroblast independent growth of primary human B lymphocytes, whereas an EBV recombinant which expresses LMP1 with only the first 44 amino acids of the carboxy terminal cytoplasmic domain can be grown on fibroblasts.  
25 The interaction between LMP1 and LAP1 is likely to be mediated by hydrophilic residues of the TRAF domain since the first 44 amino acids of the LMP1 carboxy terminal domain are remarkably hydrophilic. The induction of EBI6 by latent EBV infection is also consistent with an  
30 important role of EBI6 in EBV-mediated growth transformation of B lymphocytes.

The six markedly hydrophobic transmembrane domains of LMP1 enable it to aggregate in the plasma membrane and to present aggregated cytoplasmic domains to the TRAFs  
35 (Figure 6B). In presenting aggregated TRAF interacting

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domains, LMP1 mimics TNF-receptor aggregation which appears to be essential for signal transduction (Engelmann, et al., 1990; Loetscher, et al., 1991; Pennica, et al., 1992; Tartaglia and Goeddel, 1992).

5 Receptor crosslinking or LMP1 expression probably bring TRAF and associated molecules in close proximity creating a second messenger signal perhaps mediated by the receptor associated serine threonine kinases (Darnay, et al., 1994a; Darnay, et al., 1994b; VanArsdale and Ware,  
10 1994). Since LMP1 constitutively aggregates LAP1 and EBI6 in oligomeric complexes at plasma membrane patches these complexes could constitutively activate growth signals and NF- $\kappa$ B in the absence of extracellular stimuli. LMP1 signaling via TRAF molecules thus may proceed  
15 independently of TNF receptor molecules. Alternatively, LMP1 may stabilize the interaction of TNF family receptor-TRAF aggregates in constitutively active plasma membrane complexes. In fact, some evidence favors the latter alternative in that lymphotoxin- $\alpha$  (TNF $\beta$ ) is an  
20 autocrine growth factor for EBV-transformed lymphoblastoid cell lines (Estrov, et al., 1993; Gibbons, et al., 1994). Further, expression of the full range of EBV latent infection associated proteins in Burkitt' lymphoma cell lines induces TNF $\beta$  and the p80  
25 receptor (Gibbons, et al., 1994). Moreover, antagonistic antibodies to the p60 TNF receptor have a negative growth effect in such cells (Gibbons, et al., 1994). The LMP1 cytoplasmic carboxy terminal domain and TNFR family members could even interact with different domains of the  
30 same LAP1 molecule since there is no obvious homology between the LMP1 cytoplasmic carboxy terminus and the cytoplasmic domains of TNFR family members.

The induction of EBI6 by latent EBV infection and the association of EBI6 with LMP1 in B lymphoblasts are  
35 also evidence of an important role for EBI6 in EBV

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mediated B lymphocyte growth transformation. The interaction appears to be less direct than with LAP1 and may be mediated by another as yet unidentified human RING finger TRAF.

- 5           TNF $\alpha$  and CD40 ligand are well known mediators of growth of B lymphocytes and of other cell types that are targets for LMP1 transforming effects (Noelle, et al., 1992; Boussiotis, et al., 1994). In fact CD40 ligation and IL4 treatment are sufficient to sustain the
- 10 proliferation of primary B lymphocytes in vitro for several months and the cells are phenotypically similar to EBV transformed lymphocytes (Saeland, et al., 1993; Banchereau, et al., 1994; Galibert, et al., 1994). The LT $\beta$ R is expressed on epithelial cells; while basal
- 15 epithelial cells and anaplastic nasopharyngeal carcinoma (NPCs) cells also express high levels of CD40 (Busson et al., 1988; Young, et al. 1989). LMP1 through constitutive direct interaction with LAP1, may amplify or usurp LT $\beta$ R and CD40 signal transduction and
- 20 constitutively promote cell growth. NPC is tightly associated with EBV and LMP1 is frequently expressed in the tumor cells (Brooks, et al., 1992). Hodgkin's disease is another EBV associated malignancy in which LMP1 is expressed (Herbst, et al., 1991). CD40, TNF
- 25 receptors and the related CD30 receptor are up regulated in Hodgkin's disease cells (Froese, et al., 1987; Pfreundschuh, et al., 1989; Carde, et al., 1990; O'Grady, et al., 1994; Trumper, et al., 1994). Therefore a potentially important consequence of the demonstrated
- 30 interaction between LAP1 and LMP1 is that inhibitors of that interaction may affect the growth or development of these LMP1 associated malignancies.

In interacting with components of receptor signaling, LMP1 is somewhat similar to BPV E5 which

35 dimerizes in the plasma membrane, presumably through

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hydrophobic interactions, and activates receptors for EGF, PDGF, or CSF-1 (Martin, et al., 1989; Petti, et al., 1991; Petti and DiMaio, 1992). E5 binds a component of vacuolar H<sup>+</sup>-ATPases and this may affect receptor  
5 recycling (Goldstein, et al., 1991).

We found that LAP1 or EBI6 localize to vesicle like structures in the cytoplasm and are localized to the plasma membrane by LMP1 expression in the same cells. See, Figures 4A-4P, described below. Further, LMP1  
10 localizes EBI6 to the plasma membrane despite its inability to directly interact with EBI6, indicating that there is an abundance of another TRAF, perhaps human TRAF2 or related molecules in cells that can intermediate between LMP1 and EBI6. This raises the possibility that  
15 the TRAFs may have a role as regulators of vesicle formation or transport which may be related or unrelated to their role in TNF receptor family signaling.

The interaction of LMP1 with TNF receptor signaling pathways may also be important in enabling EBV  
20 infected cells to evade host defense mechanisms in latent or lytic EBV infection. LMP1 is one of the few EBV genes expressed in both phases of the virus life cycle (Mann, et al., 1985; Rowe, et al., 1992). Several virus families appear to specifically target the TNF/lymphot-  
25 oxin pathways presumably to avoid these immune cell mediators of cytotoxicity. Pox viruses produce soluble versions of the 80 kDa TNF receptor (Smith et al., 1991; Massung, et al., 1993). Proteins encoded by the adenovirus E3 region block the apoptotic function of TNF  
30 (Gooding, 1992) and HIV utilizes NF-kB activating signals induced by TNF signaling to enhance transcription (Poli, et al., 1990). The binding of EBV LMP1 to LAP1/EBI6 may effectively compete with normal LAP1 binding to the 60kDa TNF receptor, blocking the induction of cell death  
35 mediated by that receptor (Tartaglia, et al., 1993b) or

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### III. Screens For Inhibitors of TRAF-Mediated Functions

As detailed elsewhere, our invention particularly enables screens for inhibitors TRAF-mediated signal pathways.

#### 5                   1. In vitro screens

10                   In vitro screens may involve immobilizing one member of the interacting pair (or a relevant fragment thereof) on a substrate (e.g., the wells of a microtitre plate or beads used in a column). The immobilized member  
15 is then contacted with the other member of the interacting pair, which may be labeled. Binding is detected by detecting label associated with the substrate after washing unbound label away. In the absence of an inhibitor, this protein/protein interaction will yield  
20 bound label. Inhibitors of the protein-protein interaction will reduce the amount of bound label. Alternatively, competitive binding formats may be used, in which both binding partners are presented in the presence of candidate inhibitors, primarily competitive  
25 binding inhibitors.

Those skilled in the art will appreciate that there can be a large number of variations in the details of the above-described format. For example, polypeptide fragments can be attached to microtitre wells or beads by  
25 several well-known techniques. Labels including radioactive,



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fluorescent, and enzymatic labels may be used. Alternatively, protein/protein interaction can be measured, electrically, e.g., using the BIOCORE® apparatus, (Pharmacia). Similarly, association between  
5 glutathione and glutathione binding polypeptides such as GST can be used to detect association of two proteins, as described in Figures 5A and 5B.

Those skilled in the art will understand that there are a large number of detailed formats for  
10 performing such *in vitro* screens. For example, optical systems, such as Amersham's fluorescent pair (inhibitor-coupled) readout, may be used to detect interactions that bring fluorescent pair members in association so as to generate a fluorescent signal. BIOCORE's electrical  
15 signal generating apparatus can be used to detect direct interaction.

Screening assays for inhibitors of TRAF interactions are based on procedures for detecting binding interactions, which then serve as controls for  
20 screens in which the candidate inhibitor is added. Procedures for detecting binding interactions may be carried out using recombinant receptor proteins produced by engineered cells.

Candidate ligands may be purified (or  
25 substantially purified) molecules or the ligand may be one component of a mixture of ligands (e.g., an extract or supernatant obtained from cells. The ligand may also be identified by testing progressively smaller subsets of the ligand pool (e.g., produced by standard purification  
30 techniques, e.g., HPLC or FPLC) until a single ligand is finally demonstrated to modulate the activity in question. Candidate ligands include peptide as well as non-peptide molecules.

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Alternatively, a ligand (and an inhibitor) may be identified by its ability to bind using affinity chromatography. Recombinant binding partner is purified by standard techniques, from cells engineered to express  
5 it. The recombinant partner immobilized on a column (e.g., a Sepharose column or a streptavidin-agarose column by immunoaffinity methods) and a solution containing one or more candidate ligands is passed through the column. Again, candidate ligands include  
10 peptide as well as non-peptide molecules. A ligand specific for TRAF is immobilized on the column (because of its interaction with the TRAF). To isolate the ligand, the column is first washed to remove non-specifically bound molecules, and the ligand of interest  
15 is then released from the column and collected.

Ligands isolated from cells or biological fluids by the above methods (or any other appropriate method) may, if desired, be further purified (e.g., by high performance liquid chromatography). Once isolated in  
20 sufficiently-purified form, a novel peptide or non-peptide ligand may be partially sequenced (by standard amino acid sequencing techniques). From this partial amino acid sequence, a partial nucleic acid sequence is deduced which allows the preparation of primers for PCR  
25 cloning of the ligand gene. The TRAF, LMP1 or TNFR cytoplasmic segment which mediates interaction can be immobilized on a BIOCORE® plate and agents that inhibit interaction can be detected.

To immunologically detect a TRAF-binding molecule  
30 on the Western blot, a typical competitive antibody binding procedure can be employed, using an alkaline phosphatase-based detection protocol. Isolation of the TRAF genes also facilitates the identification of molecules which bind thereto and which may be useful as  
35 therapeutics, by providing ready sources of the full

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molecules and any desired fragments thereof, using standard recombinant DNA expression techniques.

## 2. In vivo screens

In an alternative system, cultured cells may be used to detect association of two proteins, as exemplified by the yeast two hybrid system described in more detail below. The presence of  $\beta$ -galactosidase activity indicates association of a GAL4 activating domain and a GAL4 binding domain. These two domains can be functionally associated by fusing one to a TRAF domain and the other to a TRAF-associating domain, as described below.

Other *in vivo* systems include cultures of EBV-infected cells. For example, EBV-immortalized lymphoblastoid cells lines (LCL's) may be co-cultivated with primary B lymphocytes. Lytic infection is induced (e.g., with phorbol ester). Second generation LCL's are then recovered. Cell growth of the recovered cells and the presence of EBV DNA in those cells are measured as indicative of LMP1-mediated activities.

As noted elsewhere, candidate inhibitors include polypeptide fragments of LMP1, and LAP1 and EBI6. Inhibitor fragments may be used to design and produce non-peptide drugs that retain the inhibitory function. Candidate non-peptide drugs then may be screened as described above.

## Brief Description of the Drawings

We will briefly describe the Drawings and then we will describe the specific examples related thereto. Figure 1 depicts alignment of the amino acid sequences of human EBI6 and LAP1 and murine TRAF1 and TRAF2.

Figures 2A-2D are RNA blots described below.

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Figure 3 shows intracellular association of LMP1 with LAP1 or EBI6 in transfected BJAB, non-EBV-infected, B lymphoma cells.

Figures 4A-4P are photos showing subcellular localization of LAP1 and EBI6 in the presence or absence of LMP1.

Figures 5A-5C show association of LAP1 and EBI6 with TNFR related proteins.

Figures 6A and 6B are schematic representations of TRAF mediated signal transduction.

#### Examples

I. A yeast two hybrid screen reveals proteins that interact with the LMP1 carboxy terminal cytoplasmic domain.

DNA encoding the 200 amino acid LMP1 carboxy terminal cytoplasmic domain was fused in frame to the GAL 4 DNA binding domain for use as bait in a yeast two hybrid screen for cDNAs that encode interactive proteins. The GAL 4 activating domain was fused to cDNAs made from RNA from EBV transformed B lymphocytes (Durfee, et al., 1993). Of  $5 \times 10^5$  transformants which were tested for growth in the absence of tryptophane, leucine and histidine and in the presence of 25mM 3-aminotriazole, 147 colonies showed at least moderate growth and were analyzed for  $\beta$ -galactosidase expression. Two clones were strongly positive for  $\beta$ -galactosidase, scoring higher than 8 units in a standard  $\beta$ -galactosidase assay, whereas the rest of the clones had nearly background levels of  $\beta$ -galactosidase activity (less than 0.04 units). The GAL 4 activating domain-cDNA gene fusions from these two clones did not interact with GAL4 DNA-binding domain fusions to p53, to pRB, to lamin, or to yeast SNF1 protein indicating specificity for the LMP1 cytoplasmic carboxy terminus.

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From the sequence of the complete open reading frame, full length LAP1 has an amino terminal RING finger metal binding motif and a carboxy terminal domain that begins with an extended coiled coil motif (Figure 1).

- 5 The carboxy terminal LAP1 domain (amino acids 302-568) has collinear 45% amino acid identity to the "TRAF" homology domain of the recently identified murine tumor necrosis factor (TNF) receptor associated proteins, TRAF1 and TRAF2 (Rothe, et al., 1994) (Figure 1). LAP1 is  
10 similar to TRAF2 in having an amino terminal RING finger motif but is only 27% identical to TRAF2 overall. The longest open reading frame identified in the alternatively spliced LAP1 mRNA encodes for a polypeptide that initiates at methionine codon 350 within the coiled  
15 coil motif of full length LAP1 and includes the rest of the TRAF domain. Since amino acids 345-568 of LAP1 interact strongly with the LMP1 carboxy terminal cytoplasmic domain (Table 1), the protein encoded by the spliced LAP1 mRNA could positively or negatively modulate  
20 interactions of LAP1 or LMP1.

II. The carboxy terminal 223 amino acids of LAP1 interact strongly with the membrane proximal 44 amino acids of the carboxy terminal cytoplasmic domain.

- 25 The full LMP1 cytoplasmic carboxy terminus (amino acids 187-386) interacts strongly with the LAP1 carboxy terminal 386 or 223 amino acids, although the interaction with the LAP1 223 amino acid carboxy terminal domain may be somewhat weaker (Table 1). The apparently essential  
30 membrane proximal 44 amino acids of LMP1 (amino acids 188 to 231) interact strongly with the LAP1 carboxy terminal 386 or 223 amino acids (Table 1). Thus, the LMP1 membrane proximal 44 amino acids and the LAP1 carboxy terminal 223 amino acids encompass major components of  
35 the LMP1-LAP1 interface. The apparently essential role

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of the membrane proximal 44 amino acids of LMP1 in transformation genetically links the LMP1-LAP1 biochemical interaction to LMP1 mediated transformation.

### III. Plasmid construction

5 The following genetic constructions were used to clone LAP1 and EBI6 using the yeast two hybrid system.

The GAL 4 DNA binding domain (G4DBD) fusions were constructed in vector pAS2 (Harper, et al., 1993).

10 G4DBDLMP1(187-386) was constructed by polymerase chain reaction (PCR) mediated amplification of the LMP1 cDNA fragment encoding amino acids 187-386 using oligos L1-5PCR (5'-CGCGGATCCATGGACAACGACACAGTG-3') and L1-4PCR (5'-CGCGGATCCTTAGTCATAGTAGCTTAG-3') followed by cloning into the BamHI site of pAS2. G4DBDLMP1(187-231) was

15 constructed by PCR-amplification of the LMP1 cDNA fragment encoding amino acids 187-231 using oligos L1-5PCR and LCA231 (5'-CGCGGATCCTTAGGCTCCACTCAGAGCAG-3') followed by cloning into the BamHI site of pAS2.

G4DBDLAP1(12-568) was constructed by isolating the  
20 BssHII-BamHI fragment of LAP1 cDNA from pSG5LAP1, blunt-ending it using T4 DNA polymerase and subcloning it into the SmaI site of pAS1. G4TADEBI6(53-416) was constructed by subcloning the BglII fragment of EBI6 cDNA into the BamHI site of pACTII (a kind gift of S. Elledge).

25 G4TADEBI6(53-416) encodes for an in frame fusion of EBI6 amino acids 53-416 to the acidic transactivating domain of GAL 4. G4TADLAP1(183-568) and G4TADLAP1(345-568) were isolated from the two-hybrid screening. cDNA inserts from clones were subcloned into the EcoRI site of plasmid  
30 pSG5 for sequencing analysis. pSG5 subclones of cDNA clones were spliced at the NruI site to generate full length LAP1 expressing construct pSG5LAP1. The EcoRI insert of  $\lambda$ gt10 clone EBI6 was subcloned into plasmid pBluescript for sequencing analysis. pSG5FLAGLAP1 and

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pSG5FLAGEBI6 were constructed in vector pSG5 by placing through PCR a FLAG-encoding DNA fragment right after the initiator AUG codon.

#### IV. Subtractive hybridization

- 5 Construction of the  $\lambda$ gt10 cDNA library from the EBV-positive cell line BL41/B95-8 was previously described (Birkenbach, et al., 1993). Subtractive hybridization and homology screening of a  $\lambda$ gt10 library was done as described before (Birkenbach, et al., 1993).

#### 10 V. Yeast two-hybrid screening

- Reagents necessary for culturing yeast were bought from BIO101. Yeast transformation was performed according to the method of Schiestl and Geitz (Schiestl and Gietz, 1989). The yeast strain Y190 (Durfee, et al., 15 1993) was transformed with plasmid construct G4DBDLMP1(187-386), and transformants were selected on SC-Trp plates. A single colony was picked and the expression of the LMP1 fusion protein was verified by Western blotting using the S12 anti-LMP1 monoclonal 20 antibody. The G4DBDLMP1(187-386) transformant was subsequently transformed with a cDNA library constructed previously from an EBV-transformed lymphoblastoid cell line (Durfee, et al., 1993) and selection was done on SC media lacking tryptophan, leucine and histidine in the 25 presence of 25 mM 3-aminotriazole (Sigma) as previously described (Durfee, et al., 1993). Colonies that showed moderate to intense growth were streaked on SC-Trp,Leu,His plates containing 50 mM 3-aminotriazole and tested for  $\beta$ -galactosidase expression by a filter lift 30 assay (Breen and Nasmyth, 1985). For quantitation of lacZ expression, yeast clones were grown in appropriate selective media to OD<sub>600</sub> of 0.5-1.2 and assayed for  $\beta$ -galactosidase activity using o-nitrophenyl- $\beta$ -D-galactoside (ONPG) and standard conditions as previously 35 described (Breen and Nasmyth, 1985).  $\beta$ -galactosidase

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units were expressed as  $(1000A_{415})/(\text{assay time in minutes})(\text{cell culture volume in milliliters})(\text{cell culture optical density at 600 nm})$ . Library derived plasmids were recovered by transformation of competent bacteria with total yeast DNA preps followed by selection for ampicillin resistance as previously described (Ausubel, et al., 1987).

#### VI. Northern Blots

As shown in Figures 2A-2D, Northern blots containing polyA<sup>+</sup> RNA (2  $\mu$ g per lane) from eight human tissues were purchased from Clontech. RNA was prepared from EBV-positive (BL41/B95-8) or EBV-negative (BL41) Burkitt's lymphoma cell lines and a lymphoblastoid cell line (IB4) as previously described (Birkenbach, et al., 1993). cDNA probes were labeled by random hexanucleotide priming (Stratagene) using  $^{32}\text{P}$ -dCTP. The RNA blots were hybridized to  $^{32}\text{P}$ -labeled cDNA probes, under high stringency conditions as described (Mosialos, et al., 1994). Northern blot filters were exposed to autoradiography film or processed by phosphorimager analysis.

Specifically in Figures 2A and 2B, the RNA is poly(A<sup>+</sup>)RNA from human tissues. In Figure 2C, the RNA is poly(A<sup>+</sup>)RNA from cell lines, and, in Figure 2D, the RNA is total cell line RNA. The blots were hybridized to LAP1 (2A and 2C) or EBI6 (2B and 2D) probes, shown below the blot. The origin of RNA is shown above each lane with the following designations -- PA: pancreas, KI: kidney, SM: skeletal muscle, LI: liver, LU: lung, PL: placenta, BR: brain, HE: heart. Size markers are to the left of each blot and arrows indicate the position of specifically and consistently detected mRNAs. The LAP1 probe detected 2.8 and 1.8 Kb RNAs whereas EBI6 probe detected a 2.6 Kb RNA. The high molecular weight bands were not consistently detected in other northern blots



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with these probes. LAP1 (2C) and EBI6 (2D) mRNAs were also detected in RNA from EBV infected BL41 (BL41/B95-8), EBV negative BL41 (BL41), and EBV transformed (IB4) cells. An actin probe (ACTIN) indicates the relative amounts of RNA in Figures 2C and 2D.

**VII. Immunoprecipitations, Western Blotting and immunofluorescence**

The following general technique illustrates to a method for determining intracellular protein-protein interaction. BJAB cells were electroporated at 220 V and 960  $\mu$ F in 400  $\mu$ l of RPMI-1640 medium containing 10% fetal calf serum. Approximately 20 hours post-transfection cells were lysed for 30 min on ice in 0.5% NP-40 lysis buffer containing 50 mM HEPES (pH 7.4), 250 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM PMSF, 2  $\mu$ g/ml aprotinin, 2  $\mu$ g/ml pepstatin A and 2  $\mu$ g/ml leupeptin. Cell debris were removed by centrifugation at 10,000 X g for 10 min at 4°C. The cell lysates were precleared with protein G-sepharose beads for 1 hour at 4°C. The primary antibody was then added for 1 hour at 4°C and immunoglobulin complexes were collected on protein G-sepharose beads for 1 hour at 4°C. The beads were then washed six times with 1ml of lysis buffer each time and protein complexes were recovered by boiling in SDS sample and analyzed by SDS-PAGE. Western blotting was done using standard techniques as previously described (Mosialos, et al., 1994).

Indirect immunofluorescence analysis on transfected cells was done approximately 18 to 20 hours post-transfection as previously described (Mosialos, et al., 1994).

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**VIII. Intracellular association of LMP1 with LAP1 or EB16 in transfected BJAB, non-EBV-infected, B lymphoma cells.**

BJAB cells ( $10 \times 10^6$  cells per transfection) were  
5 electroporated with plasmids expressing the proteins  
indicated by + at the bottom of the figure.  
Approximately 20 hours post transfection  $4 \times 10^6$  cells  
from each transfection were lysed and subjected to  
immunoprecipitation with 10  $\mu$ g of M2 anti-FLAG monoclonal  
10 antibody (Kodak). Results are shown in Figure 3.  
Equivalent cell lysates obtained before  
immunoprecipitation (lanes 1-4) and immunoprecipitated  
material (lanes 5-8) were analyzed by SDS polyacrylamide  
electrophoresis on a 7.5% gel, transferred onto  
15 nitrocellulose and subjected to western blot analysis  
using rabbit anti-LMP1 polyclonal antisera (Hennessy et  
al., 1984) and  $^{125}$ I-labeled protein A followed by  
autoradiography. The position of LMP1 is shown by the  
arrow and molecular weight markers are shown on the right  
20 side of the panel. LMP1 was readily coimmunoprecipitated  
with FLAG1AP1 or FLAGEBI6 (lanes 6 and 7). No detectable  
LMP1 was seen in anti-FLAG immunoprecipitations from  
cells cotransfected with pSG5LMP1 and either vector  
control (pSG5) or a construct expressing a FLAG-tagged  
25 EBNA2 (FLAGE2, lanes 5 and 8).

**IX. Production and purification of GST fusion proteins**

The cytoplasmic domains of the p80 and p60 TNF  
receptors were amplified from the corresponding cDNAs by  
PCR and were cloned in-frame into the pGEX-4T-1  
30 expression vector (Pharmacia) using the BamHI and XhoI  
restriction sites for the p60 TNF receptor and the EcoRI  
and XhoI sites for the p80 TNF receptor. Expression and  
purification of GST-fusion proteins were performed  
essentially as described previously (Smith and Johnson,  
35 1988). Fusion protein concentrations of 3-5 mg per  
milliliter of glutathione-agarose beads (Pharmacia) were

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5 routinely obtained. In vitro translations were done using the rabbit reticulocyte TNT coupled in vitro transcription translation system (Promega) according to manufacturer's protocol. In vitro translated proteins  
10 were diluted with binding buffer (PBS containing 0.1% NP-40, 0.5 mM DTT, 10% glycerol, 1 mM PMSF and 2 µg/ml aprotinin) and precleared with glutathione beads for 45 min at 4°C. GST or GST fusion proteins bound to glutathione beads were then incubated with in vitro  
15 translated proteins for 1 hour at 4°C. The beads were subsequently washed 5 times with 0.5 ml of binding buffer each time and bound proteins were recovered by boiling in SDS sample buffer and analyzed by SDS-PAGE.

**X. Subcellular localization.**

15 In Figures 4A-4P, the intracellular distribution of FLAG-tagged LAP1 and EBI6 was determined by indirect immunofluorescence using M2 anti-FLAG monoclonal antibody and rabbit anti LMP1 polyclonal antisera. BJAB cells were transfected with FLAGLAP1 (Figures 4A, 4B and 4E-4J)  
20 or FLAGEBI6 (Figures 4C, 4D and 4K-4P) expressing constructs in the presence of vector pSG5 (Figures 4A-4D) or pSG5LMP1 (Figures 4E-4P). M2 anti-FLAG reactivity was visualized with a FITC-conjugated goat anti-mouse secondary antibody (Figures 4A, 4C, 4E, 4H, 4K, 4N).  
25 LMP1 was detected with a Texas Red-conjugated goat anti rabbit secondary antibody (Figures 4F, 4I, 4L, 4O). Phase contrast pictures are shown in Figures 4B, 4D, 4G, 4J, 4M and 4P. M2 and anti-LMP1 antibodies did not show any reactivity in untransfected cells. No cross-  
30 reactivity was observed between M2 and the goat anti-rabbit secondary antibody or between the rabbit anti-LMP1 and goat anti-mouse secondary antibody (data not shown).

**XI. Association of LAP1 and EBI6 with TNFR related proteins.**

35 In Figures 5A-5C, we demonstrate association of LAP1 and EBI6 with the cytoplasmic domains of several

- 35 -

TNFRs. For example, the cytoplasmic domains of the p60 and/or p80 TNFR were constructed as fusion proteins with GST and bound to glutathione beads. These cytoplasmic domains thus bound were incubated with <sup>35</sup>S-methionine

5 labeled LMP-1, LAP1 or EBI6 translated *in vitro* (5 $\mu$ l of reaction mix) and the fraction bound to glutathione beads was analyzed on a 8.5% SDS polyacrylamide gel and processed by a phosphorimager. Coomassie blue staining of the gel demonstrated the presence of approximately

10 equivalent amounts of GST or GST-fusion proteins. In 5B, glutathione beads containing cytoplasmic domains of p60 (lane 3), p80 (lane 4), Fas (lane 5), CD40 (lane 6), LT $\beta$ R (lane 7) expressed as GST fusion proteins or GST (lane 2) were incubated with <sup>35</sup>S-methionine labeled LAP1 (2 $\mu$ l of *in*

15 *vitro* translation reaction mix were used per reaction) as in Figure 5A, and analyzed by SDS-PAGE and autoradiography (2 hr exposure). Two  $\mu$ l of *in vitro* translated LAP1 were analyzed in lane 1. Figure 5C shows co-immunoprecipitation of LAP1 and EBI6 with p80 TNFR in

20 cotransfected cells. BJAB cells were cotransfected with plasmids expressing the FLAG tagged proteins indicated by a + at the bottom of the figure of left untransfected (lane 7). Approximately 20 hours post-transfection the cells were lysed and lysates from 10 $\times$ 10<sup>6</sup> cells were

25 subjected to immunoprecipitation with M2 anti-FLAG monoclonal antibody. Equivalent cell lysates obtained before immunoprecipitation (lanes 4-7) and immunoprecipitated complexes (lanes 1-3) were analyzed by Western blotting using an anti-p80 TNFR antibody (Van

30 Arsdale and Ware 1994). The position of mature p80 TNFR and the immunoglobulin heavy chain (Ig) are shown by arrows. The star shows the position of a precursor form of the p80 TNFR. The p80 receptor was readily coimmunoprecipitated with FLAGLAP1 or FLAGEBI6 (lanes 1

35 and 2). No detectable p80 receptor was

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immunoprecipitated with anti-FLAG antibody from cells cotransfected with plasmids expressing p80 TNFR and FLAGEBNA2 (FLAGE2, lane 3).

#### Other Embodiments

5           As detailed above, the invention includes substantially pure proteins and polypeptides. We use the term polypeptide to refer to any peptide bond-containing molecule without limitation on size, including proteins and shorter polypeptides. A protein or polypeptide is  
10 substantially pure when it is separated from those contaminants which accompany it in its natural state. As we use the term, a protein which is chemically synthesized or produced in a cellular system different from the cell from which it naturally originates will be  
15 "substantially free" from its naturally associated components. Accordingly, substantially pure polypeptides include those derived from eukaryotic organisms but synthesized in *E. coli* or other prokaryotes.

The invention also includes a substantially pure  
20 nucleic acid which hybridizes at high stringency to a nucleic acid encoding LAP1. By "hybridizes" is meant binds to or associates with a nucleic acid of specified sequence. By the term "high stringency" is meant DNA hybridization and wash conditions characterized by  
25 relatively high temperature and low salt concentration, e.g., conditions described in Sambrook et al., 1989, *Molecular Cloning: a Laboratory Manual*, second edition, Cold Spring Harbor Press, Cold Spring Harbor, N.Y., e.g., 0.2 x SSC, 0.1% SDS at 60 °C wash conditions.

30           By "substantially pure DNA" is meant DNA that is free of the genes which flank the gene in the naturally-occurring genome of the organism from which the DNA of the invention is derived. The term therefore includes, for example, a recombinant DNA which is incorporated into  
35 a vector; into an autonomously replicating plasmid or

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virus; or into the genomic DNA of a prokaryote or eukaryote; or which exists as a separate molecule (e.g., a cDNA or a genomic or cDNA fragment produced by PCR or restriction endonuclease digestion) independent of other  
5 sequences. It also includes a recombinant DNA which is part of a hybrid gene encoding additional polypeptide sequence.

By "promoter" is meant minimal sequence sufficient to direct transcription. Also included in the invention  
10 are those promoter elements which are sufficient to render promoter-dependent gene expression controllable for cell-type specific, tissue-specific or inducible by external signals or agents; such elements may be located in the 5' or 3' regions of the native gene.

15 By "operably linked" is meant that a gene and a regulatory sequence(s) are connected in such a way as to permit gene expression when the appropriate molecules (e.g., transcriptional activator proteins) are bound to the regulatory sequence(s).

20 Degenerate variants of the nucleic acid encoding LAP1 or EBI6 and other polypeptides described above are also within the invention. Degenerate variants are nucleic acids which encode a polypeptide with the amino acid sequence of LAP1 or EBI6, but differ in nucleotide  
25 sequence from the cDNA sequences disclosed herein.

As used herein, the term "substantially pure" describes a protein or polypeptide, which has been separated from components which naturally accompany it. Typically, a protein or polypeptide is substantially pure  
30 when at least 10%, more preferably at least 20%, more preferably at least 50%, more preferably at least 60%, more preferably at least 75%, more preferably at least 90%, and most preferably at least 99%, of the total material (by volume, by wet or dry weight, or by mole per  
35 cent or mole fraction) in a sample is the protein or

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polypeptide of interest. Purity can be measured by any appropriate method, e.g., in the case of polypeptides by column chromatography, polyacrylamide gel electrophoresis, or high pressure liquid chromatographic (HPLC) analysis.

The invention also includes a biologically active fragment of LAP1 or EBI6. By the term "biologically active" is meant having the ability to control TRAF-mediated events such as EBV-induced phenotype traits. As used herein, the term "fragment or segment", as applied to a polypeptide, will ordinarily be at least about 5 contiguous amino acids, typically at least about 10 contiguous amino acids, more typically at least about 20 contiguous amino acids, usually at least about 30 contiguous amino acids, preferably at least about 40 contiguous amino acids, more preferably at least about 50 contiguous amino acids, and most preferably at least about 60 to 80 or more contiguous amino acids in length. Such peptides can be generated by methods known to those skilled in the art, including proteolytic cleavage of the protein, *de novo* synthesis of the fragment, or genetic engineering.

In another aspect, the invention features an antibody which specifically binds to LAP1 or EBI6.

The invention also includes homologous human LAP proteins and DNA encoding them. For example, it includes proteins more than 50% homologous to the LAP1 sequences (SEQ ID NO: 1) or active fragments thereof. "Homology", as used herein, refers to the subunit sequence similarity between two polymeric molecules, e.g., between two nucleic acid molecules, e.g., two DNA molecules, or two polypeptide molecules. When a subunit position in both of the two molecules is occupied by the same monomeric subunit, e.g., if a position in each of two DNA molecules is occupied by adenine, then they are homologous at that

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position. The homology between two sequences is a direct function of the number of matching or homologous positions, e.g., if half (e.g., 5 positions in a polymer 10 subunits in length), of the positions in two compound  
5 sequences are homologous then the two sequences are 50% homologous, if 90% of the positions, e.g., 9 of 10, are matched or homologous, the two sequences share 90% homology. By way of example, the DNA sequences 3'ATTGCC'5 and 3'TATGGC'5 share 50% homology.

10 Recombinant LAP1 or EBI6 or any fragment thereof (e.g., a biologically active domain) can be expressed using known methods. DNA sequences encoding LAP1 or EBI6 can be cloned into commercially available expression vectors and expressed in *E. coli*.

15 For example, the maltose binding protein fusion and purification system (New England Biolabs) can be used to overexpress the fusion protein. The LAP1 or EBI6 gene or cDNA can be inserted downstream and in frame of the gene encoding maltose binding protein (malE). In the  
20 absence of convenient restriction sites, PCR can be used in order to appropriately modify the cDNA sequence. This well known method can facilitate construction of the recombinant plasmid. Immediately upstream of the insertion site of the pMalE plasmid is region encoding a  
25 factor Xa cleavage site. The presence of this specific proteolytic-sensitive site allows liberation of the cloned protein from the maltose binding protein without additional amino acids attached at the N-terminus, an advantage over other methods for expressing and purifying  
30 recombinant proteins in bacteria. Using this expression system, the recombinant protein can be targeted to either the cytoplasm or periplasmic space, depending upon the presence or absence of the malE signal sequence. Purification of the fusion protein can be achieved by  
35 passing the crude cell lysate over an amylose resin



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column, to which the malE fusion protein specifically binds. The eluted pure hybrid protein can then be cleaved by factor Xa and the protein of interest purified from maltose binding protein and factor Xa by standard  
5 column chromatography.

Other expression systems, e.g., the glutathione-S-transferase gene fusion system (Pharmacia), may also be used to express all or part of the LAP1 or EBI6 proteins. In this system, TRAF DNA sequences may be cloned into the  
10 appropriate vector, and fusion proteins expressed in *E. coli*. Purification of the resulting recombinant proteins is accomplished by standard column chromatography using glutathione Sepharose 4B beads.

Alternatively, LAP1 or EBI6 can be expressed using  
15 a eucaryotic expression system. Expression vectors and eucaryotic cells suitable for expressing recombinant proteins (e.g., mammalian cells, insect cells, yeast cells) are also well known in the art.

#### Antibody Production and Western Blotting

20 In order to identify the LAP1 or EBI6 polypeptide in cellular extracts and study its potential association with other molecules, antibodies which specifically bind to those proteins are useful. Synthetic peptides designed from the predicted LAP1 or EBI6 sequence and/or  
25 the purified polypeptide produced by bacterial or eucaryotic cells can be used as antigens to immunize animals for the production of polyclonal antisera using standard protocols.

Antibodies directed against specific antigens may  
30 be detected by any of several methods known to those skilled in the art, e.g., by using an Ouchterlony double diffusion assay or an enzyme-linked immunoabsorbent assay (ELISA). ELISA involves coating a substrate, e.g., well in a plastic dish, with a purified antigen. Serum to be

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tested is then added to the well. If present, antigen specific antibodies attach to the antigen coating the well. Non-binding material is washed away and a marker enzyme e.g., horse radish peroxidase or alkaline  
5 phosphatase, coupled to a second antibody directed against the antigen-specific primary antibody is added in excess and the nonadherent material is washed away. Finally the enzyme substrate is added to the well and the enzyme catalyzed conversion is monitored as indicative of  
10 presence of the antigen.

To produce monoclonal antibodies, antibody-producing cells from the challenged animal can be immortalized (e.g., by fusion with an immortalizing fusion partner) to produce monoclonal antibodies.  
15 Monoclonal antibody-producing hybridomas can then be screened for antibody binding to the polypeptide as described above.

The invention can employ not only intact monoclonal or polyclonal antibodies, but also an  
20 immunologically-active antibody fragment, for example, a Fab or (Fab)<sub>2</sub> fragment; an antibody heavy chain, an antibody light chain; a genetically engineered single-chain Fv molecule (Ladner et al., U.S. Patent No. 4,946,778); or a chimeric antibody, for example, an  
25 antibody which contains the binding specificity of a murine antibody, but in which the remaining portions are of human origin.

The LAP1- or EBI6-specific antibodies can be employed in Western analyses in order to identify  
30 recombinant clones expressing the LAP1 or EBI6 gene product.

#### Peptide therapy

The purified polypeptides can be administered in a pharmaceutically acceptable carrier, e.g., physiological  
35 saline.

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The invention includes analogs in which one or more peptide bonds have been replaced with an alternative type of covalent bond (a "peptide mimetic") which is not susceptible to cleavage by peptidases. Where proteolytic degradation of the peptides following injection into the subject is a problem, replacement of a particularly sensitive peptide bond with a noncleavable peptide mimetic will make the resulting peptide more stable and thus more useful as a therapeutic. Such mimetics, and methods of incorporating them into polypeptides, are well known in the art. Similarly, the replacement of an L-amino acid residue is a standard way of rendering the polypeptide less sensitive to proteolysis. Also useful are amino-terminal blocking groups such as t-butylloxycarbonyl, acetyl, t-butyl, succinyl, methoxysuccinyl, suberyl, adipyl, azeloyl, dansyl, benzyloxycarbonyl, fluorenylmethoxycarbonyl, methoxyazeloyl, methoxyadipyl, methoxysuberyl, and 2,4-dinitrophenyl. Blocking the charged amino- and carboxy-termini of the peptides would have the additional benefit of enhancing passage of the peptide through the hydrophobic cellular membrane and into the cell.

The polypeptides can be administered intraperitoneally, intramuscularly, subcutaneously, or intravenously.

Standard methods for intracellular delivery of peptides can be used, e.g. with liposomes. Such methods are well known to those of ordinary skill in the art. It is expected that an intravenous dosage of approximately 1 to 100  $\mu$ moles of the peptide of the invention would be administered per kg of body weight per day.

#### Gene therapy

In some cases, patients may be treated by administering the nucleic acid of the invention, such

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that the expression of recombinant polypeptide takes place in the cells, e.g., tumor cells, of the patient, such as tumor cells. The nucleic acid of the invention may be introduced into target cells of a patient by  
5 standard vectors and/or gene delivery systems. Suitable gene delivery systems include liposomes, receptor-mediated delivery systems, naked DNA, and viral vectors such as herpes viruses, retroviruses, and adenoviruses, among others.

10 For treatment of patients, a therapeutically effective amount of a nucleic acid may be administered in a pharmaceutically acceptable carrier. Dosages for the nucleic acid molecules of the invention will vary, but a preferred dosage for intravenous administration is  
15 approximately from  $10^6$  to  $10^{22}$  copies of the nucleic acid molecule.

For treatment, a therapeutically effective amount of a nucleic acid administered in a pharmaceutically acceptable carrier. A pharmaceutically acceptable  
20 carrier is a vehicle that is suitable, i.e., biologically compatible, for administration to an animal, e.g. physiological saline. A therapeutically effective amount is an amount of the nucleic acid of the invention which is capable of producing a medically desirable result in a  
25 treated animal.

As is well known in the medical arts, dosages for any one patient depends upon many factors, including the patient's size, body surface area, age, the particular compound to be administered, sex, time and route of  
30 administration, general health, and other drugs being administered concurrently. Dosages for the nucleic acid molecules of the invention will vary, but a preferred

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dosage for intravenous administration is approximately from  $10^6$  to  $10^{22}$  copies of the nucleic acid molecule.

Once improvement of the patient's condition has occurred, a maintenance dose may be administered if  
5 necessary. Subsequently, the dosage or the frequency of administration, or both, may be reduced, as a function of the symptoms, to a level at which the improved condition is retained. When the symptoms have been alleviated to the desired level, treatment should cease. Patients may,  
10 however, require intermittent treatment on a long-term basis upon any recurrence of disease symptoms.

Also included in the invention are analogues of the native protein or polypeptides. Analogs can differ from the native peptides by amino acid sequence, or by  
15 modifications which do not affect the sequence, or by both.

Preferred analogs include peptides whose sequences differ from the wild-type sequence (i.e., the sequence of the homologous portion of the naturally occurring  
20 peptide) only by conservative amino acid substitutions, preferably by only one, two, or three, substitutions, for example, substitution of one amino acid for another with similar characteristics (e.g., valine for glycine, arginine for lysine, etc.) or by one or more non-  
25 conservative amino acid substitutions, deletions, or insertions which do not abolish the polypeptide's biological activity. Table 2 lists a number of conservative amino acid substitutions. Also included are chemically synthesized peptides with modified peptide  
30 bonds or modified side chains to obtain the desired pharmaceutical properties.

Modifications (which do not normally alter primary sequence) include *in vivo* or *in vitro* chemical derivitization of polypeptides, e.g., acetylation or  
35 carboxylation. Also included are modifications of

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glycosylation, e.g., those made by modifying the glycosylation patterns of a polypeptide during its synthesis and processing or in further processing steps, e.g., by exposing the polypeptide to enzymes which affect glycosylation e.g., mammalian glycosylating or deglycosylating enzymes. Also included are sequences which have phosphorylated amino acid residues, e.g., phosphotyrosine, phosphoserine, or phosphothreonine

For treatment of localized tumors, a bio-polymer delivery system designed for the slow release of the polypeptide of the invention may be implanted in close proximity to the tumor mass. Such bio-polymer delivery systems are well known in the art (see, e.g., Folkman et al., U.S. Patent 4,164,560, herein incorporated by reference).

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Table 1. 8.3  $\beta$ -galactosidase assay of protein-protein interactions in the yeast two-hybrid system

	<u>Transformant</u>	<u><math>\beta</math>-galactosidase</u>
	<u>units</u>	
5	G4DBDLMP1(187-386)+G4TADLAP1(183-568)	56
	G4DBDLMP1(187-386)+G4TADLAP1(345-568)	8
	G4DBDLMP1(187-231)+G4TADLAP1(183-568)	5
	G4DBDLMP1(187-231)+G4TADLAP1(345-568)	5
	G4DBDLMP1(187-386)+G4TADEBI6(53-416)	0.07
10	G4DBDLAP1(12-568)+ G4TADEBI6(53-416)	0.07
	G4DBDLMP1(187-386)	0.04
	G4DBDLMP1(187-231)	0.1
	G4TADLAP1(183-568)	0.04
	G4TADLAP1(345-568)	0.1
15	G4TADEBI6(53-416)	0.04
	G4DBDLAP1(12-568)	0.05
	G4DBDNSF1+G4TADSNF4	0.8

The yeast strain Y190 was transformed with the indicated plasmids and transformants were selected on appropriate selective defined media. Isolated colonies were grown to mid to late log density and assayed for  $\beta$ -galactosidase activity as described in experimental procedures. Four individual transformants were assayed in each case and the average values of  $\beta$ -galactosidase units are shown.

25 The interaction between G4DBDLAP1 and G4TADSNF4 was used as a control (Harper et al. 1993) and scored 0.8  $\beta$ -galactosidase units or higher in different assays.

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TABLE 2

## CONSERVATIVE AMINO ACID REPLACEMENTS

	For Amino Acid	Code	Replace With
	Alanine	A	D-Ala, Gly, Aib, $\beta$ -Ala, Acp, L-Cys, D-Cys
5	Arginine	R	D-Arg, Lys, D-Lys, homo-Arg, D-homo-Arg, Met, Ile, D-Met, D-Ile, Orn, D-Orn
	Asparagine	N	D-Asn, Asp, D-Asp, Glu, D-Glu, Gln, D-Gln
	Aspartic Acid	D	D-Asp, D-Asn, Asn, Glu, D-Glu, Gln, D-Gln
	Cysteine	C	D-Cys, S-Me-Cys, Met, D-Met, Thr, D-Thr
	Glutamine	Q	D-Gln, Asn, D-Asn, Glu, D-Glu, Asp, D-Asp
10	Glutamic Acid	E	D-Glu, D-Asp, Asp, Asn, D-Asn, Gln, D-Gln
	Glycine	G	Ala, D-Ala, Pro, D-Pro, Aib, $\beta$ -Ala, Acp
	Isoleucine	I	D-Ile, Val, D-Val, AdaA, AdaG, Leu, D-Leu, Met, D-Met
	Leucine	L	D-Leu, Val, D-Val, AdaA, AdaG, Leu, D-Leu, Met, D-Met
	Lysine	K	D-Lys, Arg, D-Arg, homo-Arg, D-homo-Arg, Met, D-Met, Ile, D-Ile, Orn, D-Orn
15	Methionine	M	D-Met, S-Me-Cys, Ile, D-Ile, Leu, D-Leu, Val, D-Val
	Phenylalanine	F	D-Phe, Tyr, D-Thr, L-Dopa, His, D-His, Trp, D-Trp, Trans-3,4, or 5-phenylproline, AdaA, AdaG, cis-3,4, or 5-phenylproline, Bpa, D-Bpa
	Proline	P	D-Pro, L-I-thioazolidine-4-carboxylic acid, D-or L-1-oxazolidine-4-carboxylic acid (Kauer, U.S. Patent (4,511,390))
	Serine	S	D-Ser, Thr, D-Thr, allo-Thr, Met, D-Met, Met(O), D-Met(O), L-Cys, D-Cys
	Threonine	T	D-Thr, Ser, D-Ser, allo-Thr, Met, D-Met, Met(O), D-Met(O), Val, D-Val
20	Tyrosine	Y	D-Tyr, Phe, D-Phe, L-Dopa, His, D-His
	Valine	V	D-Val, Leu, D-Leu, Ile, D-Ile, Met, D-Met, AdaA, AdaG



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## References

- Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (1987). Current protocols in molecular biology. John Wiley & Sons.
- Baichwal, V.R., and Sugden, B. (1988). Transformation of Balb/3T3 cells by the BNLF-1 gene of Epstein-Barr virus. *Oncogene* 4, 67-74.
- 10 Banchereau, J., Bazan, F., Blanchard, D., Briere, F., Galizzi, J.P., van Kooten, C., Liu, Y.J., Rousset, F., and Saeland, S. (1994). The CD40 antigen and its ligand. *Annu. Rev. Immunol.* 12, 881-922.
- Birkenbach, M., Josefsen, K., Yalamanchili, R., Lenoir, G., and Kieff, E. (1993). Epstein-Barr virus-induced genes: first lymphocyte-specific G protein-coupled peptide receptors. *J. Virol.* 67, 2209-2220.
- Boussiotis, V.A., Nadler, L.M., Strominger, J.L., and Goldfeld, A.E. (1994). Tumor necrosis factor alpha is an autocrine growth factor for normal human B cells. *Proc. Natl. Acad. Sci. USA* 91, 7007-7011.
- 20 Breeden, L., and Nasmyth, K. (1985). Regulation of the yeast HO gene. *Cold Spring Harbor Symp. Quant. Biol.* 50, 643-650.
- 25 Brooks, L., Yao, Q.Y., Rickinson, A.B., and Young, L.S. (1992). Epstein-Barr virus latent gene transcription in nasopharyngeal carcinoma cells: coexpression of EBNA1, LMP1, and LMP2 transcripts. *J. Virol.* 66, 2689-2697.
- 30 Busson, P., Zhang, Q., Guillon, J.M., Gregory, C.D., Young, L.S., Clausse, B., Lipinski, M., Rickinson, A.B., and Tursz, T. (1992). Elevated expression of ICAM1 (CD54) and minimal expression of LFA3 (CD58) in Epstein-Barr-virus-positive nasopharyngeal carcinoma cells. *Intl. J. Cancer* 50, 863-867.
- 35 Busson et al. (1998) *Intl. Cancer J.* 42:599-606.
- Carde, P., Da Costa, L., Manil, L., Pfreundschuh, M., Lumbroso, J., Saccavini, J., Caillou, B., Ricard, M., Boudet, F., Haya, M., Diehl, V., and Parmentier, P. (1990). Immunoscintigraphy of Hodgkin's disease: in vivo use of radiolabeled monoclonal antibodies derived from Hodgkin cell lines. *Eur. J. Cancer* 26, 474.
- 40 Cohen, J.I., Wang, F., Mannick, J., and Kieff, E. (1989). Epstein-Barr virus nuclear protein 2 is a key determinant

- 49 -

of lymphocyte transformation. Proc. Natl. Acad. Sci. USA  
86, 9558-9562.

Darnay, B.G., Reddy, A.G., and Aggarwal, B.B. (1994a).  
5 Identification of a protein kinase associated with the  
cytoplasmic domain of the p60 tumor necrosis factor  
receptor. J. Biol. Chem. 269, 20299-20304.

Darnay, B.G., Reddy, S.A.G., and Aggarwal, B.B. (1994b).  
Physical and functional association of a serine-threonine  
protein kinase to the cytoplasmic domain of the p80 form  
10 of the human tumor necrosis factor receptor in human  
histiocytic lymphoma U-937 cells. J. Biol. Chem. 269,  
19687-19690.

Dawson, C.W., Rickinson, A.B., and Young, L.S. (1990).  
Epstein-Barr virus latent membrane protein inhibits human  
15 epithelial cell differentiation. Nature 344, 777-780.

Durfee, T., Becherer, K., Chen, P.-L., Yeh, S.-H., Yang,  
Y., Kilburn, A.E., Lee, W.-H., and Elledge, S.J. (1993).  
The retinoblastoma protein associates with the protein  
phosphatase type 1 catalytic subunit. Genes and Dev. 7,  
20 555-569.

Engelmann, H., Holtmann, H., Brakebusch, C., Avni, Y.S.,  
Sarov, I., Nophar, Y., Hadas, E., Leitner, O., and  
Wallach, D. (1990). Antibodies to a soluble form of a  
tumor necrosis factor (TNF) receptor have TNF-like  
25 activity. J. Biol. Chem. 265, 14497-14504.

Estrov, Z., Kurzrock, R., Poscik, E., Pathak, S.,  
Kantarjian, H.M., Zipf, T.F., Harris, D., Talpaz, M., and  
Aggarwal, B.B. (1993). Lymphotoxin is an autocrine growth  
factor for Epstein-Barr virus-infected B cell lines. J.  
30 Exp. Med. 177, 763-774.

Fahraeus, R., Rymo, L., Rhim, J.S., and Klein, G. (1990).  
Morphological transformation of human keratinocytes  
expressing the LMP gene of Epstein-Barr virus. Nature  
345, 447-449.

35 Fennewald, S., van Santen, V., and Kieff, E. (1984). The  
nucleotide sequence of a messenger RNA transcribed in  
latent growth transforming virus infection indicates that  
it may encode a membrane protein. J. Virol. 51, 411-419.

Froese, P., Lemke, H., Gerdes, J., Havensteen, B.,  
40 Schwarting, R., Hansen, H., and Stein, H. (1987).  
Biochemical characterization and biosynthesis of the Ki-1  
antigen in Hodgkin-derived and virus-transformed human B  
and T lymphoid cells. J. Immunol. 139, 2081-2087.

- 50 -

- Galibert, L., Durand, I., Banchereau, J., and Rousset, F. (1994). CD40-activated surface IgD-positive lymphocytes constitute the long term IL-4-dependent proliferating B cell pool. *J. Immunol.* 152, 22-29.
- 5 Gibbons, D.L., Rowe, M., Cope, A.P., Feldmann, M., and Brennan, F.M. (1994). Lymphotoxin acts as an autocrine growth factor for Epstein-Barr virus-transformed B cells and differentiated Burkitt lymphoma cell lines. *Eur. J. Immunol.* 24, 1879-1885.
- 10 Goldstein, D.J., Finbow, M.E., Andresson, T., McLean, P., Smith, K., Bubb, V., and Schlegel, R. (1991). Bovine papillomavirus E5 oncoprotein binds to the 16K component of vacuolar H<sup>+</sup>-ATPases. *Nature* 352, 347-349.
- 15 Gooding, L.R. (1992). Virus proteins that counteract host immune defenses. *Cell* 71, 5-7.
- 20 Hammarskjold, M.L., and Simurda, M.C. (1992). Epstein-Barr virus latent membrane protein transactivates the human immunodeficiency virus type 1 long terminal repeat through induction of NF-kappa B activity. *J. Virol.* 66, 6496-6501.
- Hammerschmidt, W., and Sugden, B. (1989). Genetic analysis of immortalizing functions of Epstein-Barr virus in human B lymphocytes. *Nature* 340, 393-397.
- 25 Harper, J.W., Adami, G.R., Wei, N., Keyomarsi, K., and Elledge, S.J. (1993). The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell* 75, 805-816.
- 30 Hennessy, K., Fennewald, S., Hummel, M., Cole, T., and Kieff, E. (1984). A membrane protein encoded by Epstein-Barr virus in latent growth-transforming infection. *Proc. Natl. Acad. Sci. USA* 81, 7201-7211.
- 35 Herbst, H., Dallenbach, F., Hummel, M., Niedobitek, G., Pileri, S., Muller-Lantzsch, N., and Stein, H. (1991). Epstein-Barr virus latent membrane protein expression in Hodgkin and Reed-Sternberg cells. *Proc. Natl. Acad. Sci. USA* 88, 4766-4770.
- 40 Hsu, M.M., Ko, J.Y., and Chang, Y.L. (1991). Elevated levels of soluble interleukin 2 receptor and tumor necrosis factor in nasopharyngeal carcinoma. *Arch. Otolaryngol. Head Neck Surg.* 117, 1257-1259.
- Izumi, K., Kaye, K., and Kieff, E. (1994). Epstein-Barr virus recombinant molecular genetic analysis of the LMP1 amino-terminal cytoplasmic domain reveals a probable

- 51 -

structural role, with no component essential for primary B-lymphocyte growth transformation. *J. Virol.* 68, 4369-4376.

- 5 Kaye, K.M., Izumi, K.M., and Kieff, E. (1993). Epstein-Barr virus latent membrane protein 1 is essential for B-lymphocyte growth transformation. *Proc. Natl. Acad. Sci. USA* 90, 9150-9154.
- 10 Kieff, E., and Liebowitz, D. (1990). Epstein-Barr virus and its replication. In *Virology*, B. N. Fields and D. M. Knipe, eds. (New York: Raven Press), pp. 1889-1920.
- Laherty, C., Hu, H., Opipari, A., Wang, F., and Dixit, V. (1992). The Epstein-Barr virus LMP1 gene product induces A20 zinc finger protein expression by activating nuclear factor,  $\kappa$ B. *J. Biol. Chem.* 267, 24157-24160.
- 15 Liebowitz, D., Wang, D., and Kieff, E. (1986). Orientation and patching of the latent infection membrane protein encoded by Epstein-Barr virus. *J. Virol.* 58, 233-237.
- 20 Loetscher, H., Gentz, R., Zulauf, M., Lustig, A., Tabuchi, H., Schlaeger, E.-J., Brockhaus, M., Gallati, H., Manneberg, M., and Lesslauer, W. (1991). Recombinant 55-kDa tumor necrosis factor (TNF) receptor. *J. Biol. Chem.* 266, 18324-18329.
- 25 Longnecker, R., Miller, C.L., Miao, X.Q., Marchini, A., and Kieff, E. (1992). The only domain which distinguishes Epstein-Barr virus latent membrane protein 2A (LMP2A) from LMP2B is dispensable for lymphocyte infection and growth transformation in vitro; LMP2A is therefore nonessential. *J. Virol.* 66, 6461-6469.
- 30 Longnecker, R., Miller, C.L., Miao, X.Q., Tomkinson, B., and Kieff, E. (1993a). The last seven transmembrane and carboxy-terminal cytoplasmic domains of Epstein-Barr virus latent membrane protein 2 (LMP2) are dispensable for lymphocyte infection and growth transformation in vitro. *J. Virol.* 67, 2006-2013.
- 35 Longnecker, R., Miller, C.L., Tomkinson, B., Miao, X.Q., and Kieff, E. (1993b). Deletion of DNA encoding the first five transmembrane domains of Epstein-Barr virus latent membrane proteins 2A and 2B. *J. Virol.* 67, 5068-5074.
- 40 Mann, K.P., Staunton, D., and Thorley-Lawson, D.A. (1985). Epstein-Barr virus-encoded protein found in plasma membranes of transformed cells. *J. Virol.* 55, 710-720.

- 52 -

- Mannick, J.B., Cohen, J.I., Birkenbach, M., Marchini, A., and Kieff, E. (1991). The Epstein-Barr virus nuclear protein encoded by the leader of the EBNA RNAs is important in B-lymphocyte transformation. *J. Virol.* 65, 6826-6837.
- Marchini, A., Tomkinson, B., Cohen, J.I., and Kieff, E. (1991). BHRF1, the Epstein-Barr virus gene with homology to Bcl2, is dispensable for B-lymphocyte transformation and virus replication. *J. Virol.* 65, 5991-6000.
- 10 Martin, P., Vass, W.C., Schiller, J.T., Lowy, D.R., and Velu, T.J. (1989). The bovine papillomavirus E5 transforming protein can stimulate the transforming activity of EGF and CSF-1 receptors. *Cell* 59, 21-32.
- Massung, R.F., Esposito, J.J., Liu, L., Qi, J., Utterback, T.R., Knight, J.C., Aubin, L., Yuran, T.E., Parsons, J.M., Loparev, V.N., Selivanov, N.A., Cavallaro, K.F., Kerlavage, A.R., Mahy, B.W.J., and Venter, J.C. (1993). Potential virulence determinants in terminal regions of variola smallpox virus genome. *Nature* 366, 748-751.
- 20 Miller, G. (1990). Epstein-Barr virus. In *Virology*, B. N. Fields and D. M. Knipe, eds. (New York: Raven Press), pp. 1921-1958.
- Mosialos, G., Yamashiro, S., Baughman, R.W., Matsudaira, P., Vara, L., Matsumura, F., Kieff, E., and Birkenbach, M. (1994). Epstein-Barr virus infection induces expression in B lymphocytes of a novel gene encoding an evolutionarily conserved 55-kilodalton actin-bundling protein. *J. Virol.* 68, 7320-7328.
- 25 Noelle, R.J., Ledbetter, J.A., and Aruffo, A. (1992). CD40 and its ligand, an essential ligand-receptor pair for thymus-dependent B-cell activation. *Immunol. Today* 13, 431-433.
- OGrady, J.T., Stewart, S., Lowrey, J., Howie, S.E., and 35 Krajewski, A.S. (1994). CD40 expression in Hodgkin's disease. *Am. J. Pathol.* 144, 21-26.
- Pallesen, G., Hamilton-Dutoit, S.J., Rowe, M., and Young, L.S. (1991). Expression of Epstein-Barr virus latent gene products in tumour cells of Hodgkin's disease. *Lancet* 40 337, 320-322.
- Pennica, D., Kohr, W.J., Fendly, B.M., Shire, S.J., Raab, H.E., Borchardt, P.E., Lewis, M., and Goeddel, D.V. (1992). Characterization of a recombinant extracellular domain of the type I tumor necrosis factor receptor:

- 53 -

evidence for tumor necrosis factor-alpha induced receptor aggregation. *Biochemistry* 31, 1134-1141.

- Petti, L., and DiMaio, D. (1992). Stable association between the bovine papillomavirus E5 transforming protein and activated platelet-derived growth factor receptor in transformed mouse cells. *Proc. Natl. Acad. Sci. USA* 89, 6736-6740.
- Petti, L., Nilson, L.A., and DiMaio, D. (1991). Activation of the platelet-derived growth factor receptor by the bovine papillomavirus E5 transforming gene. *EMBO J.* 10, 845-855.
- Pfeffer, K., Matsuyama, T., Kundig, T.M., Wakeham, A., Kishihara, K., Shahinian, A., Wiegmann, K., Ohashi, P.S., Kronke, M., and Mak, T.W. (1993). Mice deficient for the 55 kd tumor necrosis factor receptor are resistant to endotoxic shock, yet succumb to *L. monocytogenes* infection. *Cell* 73, 457-467.
- Pfreundschuh, M., Carde, P., Da Costa, L., Manil, L., Lumbroso, J.-D., Caillou, B., Boudeta, F., Ricard, M., Parmentier, C., and Saccavini, J.-C. (1989). In vivo imaging of Hodgkin's lymphomas with monoclonal antibodies. *Onkologie(Suppl. 1)* 12, 30-42.
- Poli, G., Kinter, A., Justement, J.S., Kehrl, J.H., Bressler, P., Stanley, S., and Fauci, A.S. (1990). Tumor necrosis factor a functions in an autocrine manner in the induction of human immunodeficiency virus expression. *Proc. Natl. Acad. Sci. USA* 87, 782-785.
- Reisman, D., and Sugden, B. (1986). Trans activation of an Epstein-Barr viral transcriptional enhancer by the Epstein-Barr viral nuclear antigen 1. *Mol. Cell. Biol.* 6, 3838-3846.
- Reisman, D., Yates, J., and Sugden, B. (1985). A putative origin of replication of plasmids derived from Epstein-Barr virus is composed of two cis-acting components. *Mol. Cell. Biol.* 5, 1822-1832.
- Robertson, E.S., Tomkinson, B., and Kieff, E. (1994). An Epstein-Barr virus with a 58-kilobase-pair deletion that includes BARF0 transforms B lymphocytes in vitro. *J. Virol.* 68, 1449-1458.
- Rothe, J., Lesslauer, W., Lotscher, H., Lang, Y., Koebel, P., Kontgen, F., Althage, A., Zingernagel, R., Steinmetz, M., and Bluethmann, H. (1993). Mice lacking the tumor necrosis factor receptor 1 are resistant to TNF-mediated

- 54 -

toxicity but highly susceptible to infection by *Listeria monocytogenes*. *Nature* 364, 798-802.

5 Rothe, M., Wong, S.C., Henzel, W.J., and Goeddel, D.V. (1994). A novel family of putative signal transducers associated with the cytoplasmic domain of the 75 kDa tumor necrosis factor receptor. *Cell* 78, 681-692.

10 Rowe, M., Lear, A.L., Croom-Carter, D., Davies, A.H., and Rickinson, A.B. (1992). Three pathways of Epstein-Barr virus gene activation from EBNA1-positive latency in B lymphocytes. *J. Virol.* 66, 122-131.

Saeland, S., Duvert, V., Moreau, I., and Banchereau, J. (1993). Human B cell precursors proliferate and express CD23 after CD40 ligation. *J. Exp. Med.* 178, 113-120.

15 Schiestl, R.H., and Gietz, R.D. (1989). High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier. *Curr. Genet.* 16, 339-346.

20 Smith, C.A., Davis, T., Wignall, J.M., Din, W.S., Farrah, T., Upton, C., McFadden, G., and Goodwin, R.G. (1991). T2 open reading frame from the Shope fibroma virus encodes a soluble form of the TNF receptor. *Biochem. Biophys. Res. Commun.* 176, 335-342.

25 Smith, D.B., and Johnson, K.S. (1988). Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. *Gene* 67, 31-40.

30 Sugden, B., and Warren, N. (1989). A promoter of Epstein-Barr virus that can function during latent infection can be transactivated by EBNA-1, a viral protein required for viral DNA replication during latent infection. *J. Virol.* 63, 2644-2649.

35 Swaminathan, S., Tomkinson, B., and Kieff, E. (1991). Recombinant Epstein-Barr virus with small RNA (EBER) genes deleted transforms lymphocytes and replicates in vitro. *Proc. Natl. Acad. Sci. USA* 88, 1546-1550.

Tartaglia, L.A., and Goeddel, D.V. (1992). Tumor necrosis factor receptor signaling. A dominant negative mutation suppresses the activation of the 55-kDa tumor necrosis factor receptor. *J. Biol. Chem.* 267, 4304-4307.

40 Tartaglia, L.A., Goeddel, D.V., Reynolds, C., Figari, I.S., Weber, R.F., Fendly, B.M., and Palladino, M.A.J. (1993a). Stimulation of human T-cell proliferation by

- 55 -

specific activation of the 75-kDa tumor necrosis factor receptor. *J. Immunol.* 151, 4637-4641.

- 5 Tartaglia, L.A., Rothe, M., Hu, Y.-F., and Goeddel, D.V. (1993b). Tumor necrosis factor's cytotoxic activity is signaled by the p55 TNF receptor. *Cell* 73, 213-216.

- 10 Tomkinson, B., and Kieff, E. (1992). Use of second-site homologous recombination to demonstrate that Epstein-Barr virus nuclear protein 3B is not important for lymphocyte infection or growth transformation in vitro. *J. Virol.* 66, 2893-2903.

Tomkinson, B., Robertson, E., and Kieff, E. (1993). Epstein-Barr virus nuclear proteins (EBNA) 3A and 3C are essential for B lymphocyte growth transformation. *J. Virol.* 67, 2014-2025.

- 15 Trumper, L., Jung, W., Dahl, G., Diehl, V., Gause, A., and Pfeundschuh, M. (1994). Interleukin-7, interleukin-8, soluble TNF receptor, and p53 protein levels are elevated in the serum of patients with Hodgkin's disease. *Ann. Oncol.* 5, 93-96.

- 20 VanArsdale, T.L., and Ware, C.F. (1994). TNF receptor signal transduction. Ligand-dependent stimulation of a serine protein kinase activity associated with (CD120a) TNFR60. *J. Immunol.* 153, 3043-3050.

- 25 Wang, D., Liebowitz, D., and Kieff, E. (1985). An EBV membrane protein expressed in immortalized lymphocytes transforms established rodent cells. *Cell* 43, 831-840.

- 30 Wang, D., Liebowitz, D., and Kieff, E. (1988a). The truncated form of the Epstein-Barr virus latent-infection membrane protein expressed in virus replication does not transform rodent fibroblasts. *J. Virol.* 62, 2337-2346.

- 35 Wang, D., Liebowitz, D., Wang, F., Gregory, C., Rickinson, A., Larson, R., Springer, T., and Kieff, E. (1988b). Epstein-Barr virus latent infection membrane protein alters the human B-lymphocyte phenotype: deletion of the amino terminus abolishes activity. *J. Virol.* 62, 4173-4184.

- 40 Wang, F., Gregory, C., Sample, C., Rowe, M., Liebowitz, D., Murray, R., Rickinson, A., and Kieff, E. (1990). Epstein-Barr virus latent membrane protein (LMP1) and nuclear proteins 2 and 3C are effectors of phenotypic changes in B lymphocytes: EBNA-2 and LMP1 cooperatively induce CD23. *J. Virol.* 64, 2309-2318.



- 56 -

Wilson, J.B., Weinberg, W., Johnson, R., Yuspa, S., and  
Levine, A.J. (1990). Expression of the BNLf-1 oncogene of  
Epstein-Barr virus in the skin of transgenic mice induces  
hyperplasia and aberrant expression of keratin 6. Cell  
5 61, 1315-1327.

Yates, J., Warren, N., Reisman, D., and Sugden, B.  
(1984). A cis-acting elements from the Epstein-Barr virus  
genome that permits stable replication of recombinant  
plasmids in latently infected cells. Proc. Natl. Acad.  
10 Sci. USA 81, 3806-3810.

Young, L.S., Dawson, C.W., Brown, K.W., and Rickinson,  
A.B. (1989). Identification of a human epithelial cell  
surface protein sharing an epitope with the C3d/Epstein-  
Barr virus receptor molecule of B lymphocytes. Intl. J.  
15 Cancer 43, 786-794.

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## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: Brigham & Women's Hospital
- (ii) TITLE OF INVENTION: CONTROLLING TRAF-MEDIATED SIGNALS
- (iii) NUMBER OF SEQUENCES: 3
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: Fish & Richardson
  - (B) STREET: 225 Franklin Street Suite 3100
  - (C) CITY: Boston
  - (D) STATE: MA
  - (E) COUNTRY: U.S.A.
  - (F) ZIP: 02110-2804
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30B
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER: PCT/US95/----
  - (B) FILING DATE:
- (vii) PRIOR APPLICATION DATA:
  - (A) APPLICATION NUMBER: 08/367,540
  - (B) FILING DATE: 30-DEC-1994
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: Freeman, John W.
  - (B) REGISTRATION NUMBER: 29,066
  - (C) REFERENCE/DOCKET NUMBER: 05311/014WO1
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: (617)542-5070
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  - (C) TELEX: 100254

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 2359 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (ix) FEATURE:
  - (A) NAME/KEY: CDS
  - (B) LOCATION: 151..1854
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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Met Glu Ser Ser Lys Lys Met Asp																
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Ser	Pro	Gly	Ala	Leu	Gln	Thr	Asn	Pro	Pro	Leu	Lys	Leu	His	Thr	Asp	
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CGC	AGT	GCT	GGG	ACG	CCA	GTT	TTT	GTC	CCT	GAA	CAA	GGA	GGT	TAC	AAG	270
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GAA	AAG	TTT	GTG	AAG	ACC	GTG	GAG	GAC	AAG	TAC	AAG	TGT	GAG	AAG	TGC	318
Glu	Lys	Phe	Val	Lys	Thr	Val	Glu	Asp	Lys	Tyr	Lys	Cys	Glu	Lys	Cys	
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Thr	Ala	Cys	Gln	Glu	Ser	Ile	Val	Lys	Asp	Lys	Val	Phe	Lys	Asp	Asn	
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Cys	Cys	Lys	Arg	Glu	Ile	Leu	Ala	Leu	Gln	Ile	Tyr	Cys	Arg	Asn	Glu	
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TTA	AAA	AAT	GAT	TGC	CAT	TTT	GAA	GAA	CTT	CCA	TGT	GTG	CGT	CCT	GAC	606
Leu	Lys	Asn	Asp	Cys	His	Phe	Glu	Glu	Leu	Pro	Cys	Val	Arg	Pro	Asp	
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Cys	Lys	Glu	Lys	Val	Leu	Arg	Lys	Asp	Leu	Arg	Asp	His	Val	Glu	Lys	
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GCG	TGT	AAA	TAC	CGG	GAA	GCC	ACA	TGC	AGC	CAC	TGC	AAG	AGT	CAG	GTT	702
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GTG	GTG	TCC	TGC	CCT	CAC	AAG	TGC	AGC	GTC	CAG	ACT	CTC	CTG	AGG	AGC	798
Val	Val	Ser	Cys	Pro	His	Lys	Cys	Ser	Val	Gln	Thr	Leu	Leu	Arg	Ser	
				205					210					215		

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GAG	TTG	AGT	GCA	CAC	TTG	TCA	GAG	TGT	GTC	AAT	GCC	CCC	AGC	ACC	TGT	846
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AGT	TTT	AAG	CGC	TAT	GGC	TGC	GTT	TTT	CAG	GGG	ACA	AAC	CAG	CAG	ATC	894
Ser	Phe	Lys	Arg	Tyr	Gly	Cys	Val	Phe	Gln	Gly	Thr	Asn	Gln	Gln	Ile	
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AAG	GCC	CAC	GAG	GCC	AGC	TCC	GCC	GTG	CAG	CAC	GTC	AAC	CTG	CTG	AAG	942
Lys	Ala	His	Glu	Ala	Ser	Ser	Ala	Val	Gln	His	Val	Asn	Leu	Leu	Lys	
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GAG	TGG	AGC	AAC	TCG	CTC	GAA	AAG	AAG	GTT	TCC	TTG	TTG	CAG	AAT	GAA	990
Glu	Trp	Ser	Asn	Ser	Leu	Glu	Lys	Lys	Val	Ser	Leu	Leu	Gln	Asn	Glu	
265					270					275					280	
AGT	GTA	GAA	AAA	AAC	AAG	AGC	ATA	CAA	AGT	TTG	CAC	AAT	CAG	ATA	TGT	1038
Ser	Val	Glu	Lys	Asn	Lys	Ser	Ile	Gln	Ser	Leu	His	Asn	Gln	Ile	Cys	
				285					290					295		
AGC	TTT	GAA	ATT	GAA	ATT	GAG	AGA	CAA	AAG	GAA	ATG	CTT	CGA	AAT	AAT	1086
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Lys	Leu	Lys	Glu	Leu	Asp	Lys	Glu	Ile	Arg	Pro	Phe	Arg	Gln	Asn	Trp	
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				365				370						375		
AAC	ACA	GGC	CTG	CTG	GAG	TCC	CAG	CTG	AGC	CGG	CAT	GAC	CAG	ATG	CTG	1326
Asn	Thr	Gly	Leu	Leu	Glu	Ser	Gln	Leu	Ser	Arg	His	Asp	Gln	Met	Leu	
			380					385					390			
AGT	GTG	CAC	GAC	ATC	CGC	CTA	GCC	GAC	ATG	GAC	CTG	CGC	TTC	CAG	GTC	1374
Ser	Val	His	Asp	Ile	Arg	Leu	Ala	Asp	Met	Asp	Leu	Arg	Phe	Gln	Val	
		395					400					405				
CTG	GAG	ACC	GCC	AGC	TAC	AAT	GGA	GTG	CTC	ATC	TGG	AAG	ATT	CGC	GAC	1422
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## (2) INFORMATION FOR SEQ ID NO: 2:

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- (A) LENGTH: 2380 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

## (ix) FEATURE:

- (A) NAME/KEY: CDS  
 (B) LOCATION: 76..1323

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CCT GAT GAG AAT GAG TTT CCC TTT GGG TGC CCT CCC ACC GTC TGC CAG      159
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GAC CCA AAG GAG CCC AGG GCT CTC TGC TGT GCA GGC TGT CTC TCT GAG      207
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GAG	CTG	GAG	GGG	AAG	CTG	CGT	GTG	TTT	GAG	AAC	ATT	GTT	GCT	GTC	CTC	687
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Phe	Leu	Trp	Lys	Ile	Thr	Asn	Val	Thr	Arg	Arg	Cys	His	Glu	Ser	Ala	
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Cys	Gly	Arg	Thr	Val	Ser	Leu	Phe	Ser	Pro	Ala	Phe	Tyr	Thr	Ala	Lys	
		855					860					865				
TAT	GGC	TAC	AAG	TTG	TGC	CTG	CGG	CTG	TAC	CTG	AAT	GGA	GAT	GGC	ACT	1023
Tyr	Gly	Tyr	Lys	Leu	Cys	Leu	Arg	Leu	Tyr	Leu	Asn	Gly	Asp	Gly	Thr	
	870					875					880					
GGA	AAG	AGA	ACC	CAT	CTG	TCG	CTC	TTC	ATC	GTG	ATC	ATG	AGA	GGG	GAG	1071
Gly	Lys	Arg	Thr	His	Leu	Ser	Leu	Phe	Ile	Val	Ile	Met	Arg	Gly	Glu	
885					890					895					900	
TAT	GAT	GCG	CTG	CTG	CCG	TGG	CCC	TTC	CGG	AAC	AAG	GTC	ACC	TTC	ATG	1119
Tyr	Asp	Ala	Leu	Leu	Pro	Trp	Pro	Phe	Arg	Asn	Lys	Val	Thr	Phe	Met	
				905					910					915		
CTG	CTG	GAC	CAG	AAC	AAC	CGT	GAG	CAC	GCC	ATT	GAC	GCC	TTC	CGG	CCT	1167
Leu	Leu	Asp	Gln	Asn	Asn	Arg	Glu	His	Ala	Ile	Asp	Ala	Phe	Arg	Pro	
			920					925					930			
GAC	CTA	AGC	TCA	GCG	TCC	TTC	CAG	AGG	CCC	CAG	AGT	GAA	ACC	AAC	GTG	1215
Asp	Leu	Ser	Ser	Ala	Ser	Phe	Gln	Arg	Pro	Gln	Ser	Glu	Thr	Asn	Val	
		935					940					945				

- 63 -

GCC AGT GGA TGC CCA CTC TTC TTC CCC CTC AGC AAA CTG CAG TCA CCC	1263
Ala Ser Gly Cys Pro Leu Phe Phe Pro Leu Ser Lys Leu Gln Ser Pro	
950 955 960	
AAG CAC GCC TAC GTG AAG GAC GAC ACA ATG TTC CTC AAG TGC ATT GTG	1311
Lys His Ala Tyr Val Lys Asp Asp Thr Met Phe Leu Lys Cys Ile Val	
965 970 975 980	
GAG ACC AGC ACT TAGGGTGGGC GGGGCTCCTG AGGGAGCTCC AACTCAGAAG	1363
Glu Thr Ser Thr	
GGAGCTAGCC AGAGGACTTG TGATGCCCTG CCCTTGGCAC CCAAGACCTC AGGGCACAAA	1423
GATGGGTGAA GGCTGGCTGA TCCAAGCAAG ACTGAGGGGT CGACTTCGGG CTGGCCATCT	1483
GGTTAGGATG GCAGGACGTG GGCTGGGCCC ACAAAGGCAA AGGGTCCAAG AAGGAGACAG	1543
GCAGAGCTGC TCCCCTCGCA CGGACCATGC GACACTGGGA GGCCAGTGAG CCACTCCGGC	1603
CCCGAATGTT GAGGTGGACT CTCACCAAAT GAGAAGAAAA TGGAACCAGG CTTGGAACCG	1663
TAGGACCCAA GCAGAGAAGC TCTCGGGCTA GGAAGATCTC TGCAGGGCCG CCAGGGAGAC	1723
CTGGACACAG GCCTGCTCTC TTTTCTCCA GGGTCAGAAA CAGGACCGGG TGGAAGGGAT	1783
GGGGTGCCAG TTTGAATGCA GTCTGTCCAG GCTCGTCATT GGAGGTGAAC AAGCAAACCC	1843
AGACGGCTCC ACTAGGACTT CAAATTGGGG GTTGGATTG AAGACTTTTA AGTTTCCTTC	1903
CAGCCCAGAA AGTCTCTCAT TCTAGCCTCC TGGCCCAGGT GAGTCCTAGA GCTACAGGGG	1963
TTCTGGAAC ATTCAGGAGC TTCCTGTCCT CCCAGCTCCT CACTCACCTT CAGTAACCCC	2023
CACTGGACTG ACCTGGTCCA CAGGGCACCT GCCACCCTGG GCCTGGCAGC TCAGCTTCCC	2083
AACACGCAGG AGCACACCCA GCCCCACAT CCTGTGCCTC CATCAGCTAA ACACCACGTC	2143
ACTTCATGCA GGTGAAACCC AGTCACTGTG AGCTCCCAGG TGCAGCCAGA GGCACCTCAA	2203
GAAGAAGAGG GGCATAAACT TTCCTCTTCC TGCCTAGAGG CCCCACCTTT GGTGCTTTCC	2263
AGAATCCCGT AACACCTGAT TAACTGAGGC ATCCAATTCT TTCAGCAGAC TGATCAGGAC	2323
CTCCAAGCCA CTGAGCAATG TATAACCCCA AAGGGAATTC AAAAAAAAAA AAAAAAA	2380

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 44 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein



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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Gly	Gln	Arg	His	Ser	Asp	Glu	His	His	His	Asp	Asp	Ser	Leu	Pro	His
1				5					10					15	
Pro	Gln	Gln	Ala	Thr	Asp	Asp	Ser	Gly	His	Glu	Ser	Asp	Ser	Asn	Ser
			20					25					30		
Asn	Glu	Gly	Arg	His	His	Leu	Leu	Val	Ser	Gly	Ala				
		35					40								

What is claimed is:

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1. A method of treating Epstein-Barr virus (EBV)-associated infection, cell growth or tumorigenesis by administering to EBV-infected cells a compound that inhibits TRAF/TNFR-mediated cell growth/death signal transduction.  
5
2. A medicament for treating Epstein-Barr virus (EBV)-associated infection, cell growth or tumorigenesis, said medicament comprising a compound that inhibits TRAF/TNFR-mediated cell growth/death signal transduction.
- 10 3. A method of making a medicament for treating Epstein-Barr virus (EBV)-associated infection, cell growth or tumorigenesis, said medicament comprising a compound that inhibits TRAF/TNFR-mediated cell growth/death signal transduction.
- 15 4. A method of treating Epstein-Barr virus (EBV)-associated infection, cell growth or tumorigenesis by administering to EBV-infected cells a compound that inhibits interaction between EBV LMP1 and a Tumor necrosis factor Receptor Associated Factor (TRAF)  
20 protein.
5. A medicament for treating Epstein-Barr virus (EBV)-associated infection, cell growth or tumorigenesis, said medicament comprising a compound that inhibits interaction between EBV LMP1 and a Tumor necrosis factor  
25 Receptor Associated Factor (TRAF) protein.
6. A method of making a medicament for treating Epstein-Barr virus (EBV)-associated infection, cell growth or tumorigenesis, said medicament comprising a compound that inhibits interaction between EBV LMP1 and a

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Tumor necrosis factor Receptor Associated Factor (TRAF) protein.

7. The method of claim 4 or claim 6, or the medicament of claim 5, in which the compound is a polypeptide that interacts with EBV LMP1 protein.

8. The method or medicament of claim 7 in which the polypeptide includes an LMP1-interacting TRAF domain.

9. The method or medicament of claim 7 in which the polypeptide interacts with a region of the LMP1 carboxy terminus between amino acids 188 and 386.

10. The method or medicament of claim 7 in which the polypeptide interacts with the LMP1 sequence that extends from Gly188 to Ala231: G Q R H S D E H H H D D S L P H P Q Q A T D D S G H E S D S N S N E G R H H L L V S G A (SEQ ID NO:3).

11. The method or medicament of claim 8 in which the polypeptide includes a LMP1-interacting domain of a human TRAF protein.

12. The method or medicament of claim 11 in which the polypeptide includes a LMP1-interacting domain of LAP1.

13. The method or medicament of claim 12 in which the polypeptide includes a LMP1-binding domain within the LAP1 sequence between amino acids 345 and 568 of SEQ ID NO:1.

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14. The method of claim 4 or claim 6, or the medicament of claim 5, in which the compound is a polypeptide that interacts with a TRAF protein.

15. The method or medicament of claim 14 in which  
5 the polypeptide includes a LMP1 sequence that interacts with a TRAF protein.

16. The method or medicament of claim 15 in which the polypeptide includes a LMP1 sequence that interacts with LAP1.

10 17. The method or medicament of claim 16 in which the polypeptide includes the sequence: G Q R H S D E H H  
H D D S L P H P Q Q A T D D S G H E S D S N S N E G R H H  
L L V S G A (SEQ ID NO:3).

18. The method or medicament of claim 14 in which  
15 the polypeptide interacts with a LMP1-binding domain within the LAP1 sequence between amino acids 345 and 568 of SEQ ID NO:1.

19. The method or medicament of claim 14 in which the polypeptide includes a human LAP oligomer-forming  
20 domain.

20. The method or medicament of claim 19 in which the polypeptide includes the following LAP1 sequence  
(amino acids 309-341 of SEQ ID NO:1):

25 L R N N E S K I L H L Q R V I D S  
Q A E K L K E L D K E I R P F R

21. The method or medicament of claim 14 in which the polypeptide includes a TRAF oligomer-forming domain.

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22. The method of claim 1, claim 3, claim 4, claim 6, or the medicament of claim 2 or claim 5, in which the compound is administered to a patient characterized by one or more of the following conditions:

- 5 a) EBV infection; b) HIV infection; c) drug induced immunosuppression; d) Hodgkin's disease, e) Burkitt's lymphoma, f) a lymphoma characteristic of an immunocompromised patient, and g) nasopharyngeal carcinoma.

10 II. Controlling TRAF-Mediated TNF/TNFR Signaling

23. A method of controlling TRAF-Mediated TNF/TNFR signal transduction by administering to a TRAF-encoding cell a compound that inhibits TRAF signal transduction.

- 15 24. A medicament for controlling TRAF-Mediated TNF/TNFR signal transduction, said medicament comprising a TRAF-encoding cell a compound that inhibits TRAF signal transduction.

- 20 25. A method of making a medicament for controlling TRAF-Mediated TNF/TNFR signal transduction, said medicament comprising a TRAF-encoding cell a compound that inhibits TRAF signal transduction.

26. The method of claim 23 of claim 25, or the medicament of claim 24 in which the compound is a TRAF-  
25 interacting polypeptide.

27. The method or medicament of claim 26 in which the polypeptide includes a TRAF-interacting domain selected from the group consisting of: a) a LAP1 coiled coil domain; b) a LAP1 carboxy terminal domain extending

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from amino acids 406-568 of SEQ ID NO:1; c) an EBI6 coiled coil domain; d) an EBI6 carboxy terminal domain extending from amino acids 259-416 of SEQ ID NO:2; e) a TRAF-interacting TNFR cytoplasmic domain; f) a TRAF metal  
5 binding domain.

28. The method or medicament of claim 26 in which the compound interacts with an oligomerizing TRAF domain.

29. The method or medicament of claim 28 in which the polypeptide includes a human TRAF coiled coil  
10 domain.

30. The method or medicament of claim 26 in which the polypeptide includes the sequence (amino acids 309-341 of SEQ ID NO:1):

15           L R N N E S K I L H L Q R V I D S  
          Q A E K L K E L D K E I R P F R.

31. The method of claim 23 or claim 25, or the medicament of claim 24, in which the compound is administered to a patient with undesired lymphocyte proliferation, manifest as an autoimmune disease, e.g.,  
20 rheumatoid arthritis, Crone's disease, lupus, or to a patient characterized by drug induced immunosuppression.

### III. Purified Polypeptides, Reagents and Methods For Making Them

32. A purified polypeptide capable of controlling  
25 TRAF-Mediated TNF/TNFR signal transduction when administered to a TRAF-encoding cell.

33. The polypeptide of claim 32 in which said polypeptide is capable of inhibiting interaction between

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EBV LMP1 and a Tumor necrosis factor Receptor Associated Factor (TRAF) protein.

34. The polypeptide of claim 32 in which the polypeptide includes a LMP1-interacting TRAF domain.

5           35. The polypeptide of claim 34 in which the polypeptide includes a LMP1-interacting domain of a human TRAF protein.

36. The polypeptide of claim 35 in which the polypeptide includes the LMP1-interacting domain of LAP1.

10           37. The polypeptide of claim 36 in which the polypeptide includes a LMP1-binding domain within the LAP1 sequence between amino acids 345 and 568 of SEQ ID NO:1).

15           38. The polypeptide of claim 32 in which the polypeptide interacts with the following LMP1 sequence: G Q R H S D E H H H D D S L P H P Q Q A T D D S G H E S D S N S N E G R H H L L V S G A (SEQ ID NO:3).

39. The polypeptide of claim 32 in which the polypeptide interacts with a TRAF protein.

20           40. The polypeptide of claim 39 in which the polypeptide includes a LMP1 sequence that interacts with LAP1.

25           41. The polypeptide of claim 32 in which the polypeptide includes a domain selected from the group consisting of: a) a LAP1 coiled coil domain; b) a LAP1 carboxy terminal domain extending from amino acids 406-568 of SEQ ID NO:1; c) a LAP1 metal binding domain; d) an

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EBI6 coiled coil domain; e) an EBI6 carboxy terminal domain extending from amino acids 259-416 of SEQ ID NO:2; and f) a TRAF-interacting cytoplasmic domain of a TNFR.

42. The polypeptide of claim 32 in which the  
5 polypeptide interacts with a LMP1-binding domain within the LAP1 sequence between amino acids 345 and 568 of SEQ ID NO:1).

43. The polypeptide of claim 32 in which the polypeptide includes a TRAF oligomer-forming domain.

10 44. The polypeptide of claim 43 in which the polypeptide includes a human LAP oligomer-forming domain.

45. The polypeptide of claim 44 in which the polypeptide includes a human LAP1 oligomer-forming domain.

15 46. The polypeptide of claim 45 in which the polypeptide includes the following LAP1 sequence (amino acids 309-341 of SEQ ID NO:1):

L R N N E S K I L H L Q R V I D S  
Q A E K L K E L D K E I R P F R

20 47. The polypeptide of claim 32 in which the polypeptide includes a TRAF protein-interacting TNRF cytoplasmic domain that includes the sequence:

TX<sub>1-4</sub>EE/DX<sub>0-2</sub>K, where T, E, D, and K are standard single letter amino acid designations, X can be any amino acid,  
25 X<sub>0-4</sub> and X<sub>0-2</sub> indicate optionally, from 0-4 or 0-2 amino acid residues, respectively, and E/D indicates a single amino acid residue that is either E or D.



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48. A medicament for controlling TRAF-Mediated TNF/TNFR signal transduction when administered to a TRAF-encoding cell, said medicament comprising the polypeptide of claims 32-48.

5 49. Purified recombinant nucleic acid encoding the polypeptide of claim 32.

50. The recombinant nucleic acid of claim 49 in which the recombinant nucleic acid further comprises regulatory DNA positioned to transcribe the polypeptide  
10 encoding DNA.

51. A cell comprising the recombinant nucleic acid of claim 50.

52. A medicament for controlling TRAF-Mediated TNF/TNFR signal transduction when administered to a TRAF-  
15 encoding cell, said medicament comprising the purified nucleic acid of claim 50 or the cell of claim 51.

#### IV. Screening Techniques

53. A method of making the purified polypeptide of claim 32, comprising  
20 culturing a cell containing recombinant nucleic acid encoding the polypeptide, the recombinant nucleic acid further comprising regulatory DNA positioned to transcribe the polypeptide-encoding nucleic acid in the cell, and  
25 recovering the purified polypeptide from the cell or the culture medium.

54. A method of *in vitro* screening for substances that inhibit Tumor necrosis factor Receptor Associated Factor (TRAF) protein-related signal transduction, by:

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- a. providing a compound that includes a TRAF interacting domain; and
- b. combining the compound with a candidate inhibitor;
- 5 c. determining whether the candidate inhibitor binds the compound.

55. The method of claim 54 in which the TRAF interacting domain is a) a LMP1 domain that includes the sequence G Q R H S D E H H H D D S L P H P Q Q A T D D S  
10 G H E S D S N S N E G R H H L L V S G A (SEQ ID NO:3); b) a LMP1-interacting LAP1 domain contained within amino acids 345-586 of SEQ ID NO:1; c) a TRAF-interacting TRAF domain; f) a TNFR-interacting TRAF domain; e) a TRAF-interacting TNFR cytoplasmic domain; or f) a TRAF metal  
15 binding domain.

56. A method of *in vitro* screening for substances that inhibit interaction of LMP1 with a Tumor necrosis factor Receptor Associated Factor (TRAF) protein, by:

- a. providing a first binding partner and a  
20 second binding partner in a system that permits the binding partners to interact with each other, the first binding partner having a TRAF protein-interacting LMP1 domain, the second binding partner interacting with the LMP1 domain of the first binding partner; and
- 25 b. providing a candidate compound in the system;
- c. determining whether the candidate compound inhibits interaction of the first binding partner and the second binding partner in the system.

30 57. The method of claim 56 in which the first binding partner's TRAF-interacting domain is a LMP1 domain that includes the sequence G Q R H S D E H H H D D

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S L P H P Q Q A T D D S G H E S D S N S N E G R H H L L V  
S G A (SEQ ID NO:3).

58. The method of claim 56 in which the second binding partner's LMP1-interacting domain is a LAP1 domain contained within amino acids 345-586 of SEQ ID NO:1.

59. The method of claim 56 in which the first binding partner is LMP1 and the second binding partner is LAP1.

10 60. The method of claim 56 in which one of the binding partners is immobilized on a solid phase, and the other binding partner is provided in a liquid contacting the solid phase.

61. The method of claim 56 wherein the other binding partner includes a label to permit its detection.

62. The method of claim 56 in which the solid phase is part of an electrical circuit, and binding by the other partner is detected by measuring electrical characteristics of the circuit.

20 63. A method of *in vivo* screening for substances that inhibit interaction of LMP1 with a Tumor necrosis factor Receptor Associated Factor (TRAF) protein, by:  
providing cells that express a TRAF and LMP1,  
interaction between the TRAF and LMP1 resulting in a  
25 detectable phenotypic trait.

64. The method of claim 63 in which interaction between the TRAF and the TRAF-interacting molecule is determined by detecting a phenotype of EBV infection.

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65. A method of *in vitro* screening for substances that inhibit interaction of a Tumor necrosis factor Receptor Associated Factor (TRAF) protein with a member of the tumor necrosis factor receptor family, by:

- 5           a. providing a first binding partner and a second binding partner in a system that permits the binding partners to interact with each other, the first binding partner having a TRAF protein-interacting TNFR domain, the second binding partner comprising a TRAF  
10 protein domain that interacts with the TNFR domain of the first binding partner; and
- b. providing a candidate compound in the system;
- c. determining whether the candidate  
15 compound inhibits interaction of the first binding partner and the second binding partner in the system.

66. The method of claim 65 in which the TRAF protein-interacting domain is a TNFR cytoplasmic domain that includes the sequence:  $TX_{1-4}EE/DX_{0-2}K$ , where T, E, D, and K are standard single-letter amino acid designations,  
20 X can be any amino acid,  $X_{0-4}$  and  $X_{0-2}$  indicate optionally, from 0-4 or 0-2 amino acid residues, respectively, and E/D indicates a single amino acid residue that is either E or D.

25           67. The method of claim 65 in which the TRAF protein domain is a TNFR-interacting domain contained within the LAP1 amino acid sequence 345-586 of SEQ ID NO:1 or the EBI6 amino acid sequence 259-416 of SEQ ID NO:2.

30           68. The method of claim 65 in which the TNFR protein domain is a TRAF-interacting sequence within the cytoplasmic domain of a TNFR family member.

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69. The method of claim 68 in which the TNFR family member is selected from the group consisting of p80, CD40, lymphotoxin- $\beta$ , p60, and Fas.

70. Antibodies that specifically bind to a LAP1  
5 or EBI6 immunodeterminant.

71. Antibodies raised by challenge with an antigen that contains a LAP1 or EBI6 immunodeterminant.

72. Purified human Epstein-Barr virus Induced Protein-6 (EBI6).

10 73. A purified human LAP.

74. Purified human LAP1.

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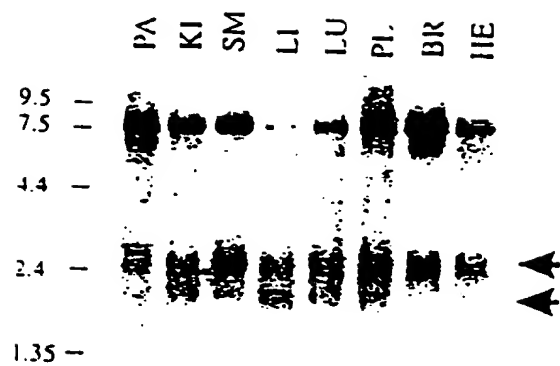
LAP1	MESSKKMDSFGALQTNPLKLHTDRSAGTPVFVPEOGGYKEKFVKT-VEDKYKCE	54
TRAF2	MAAAS-VTSPGSELELQP-----GFSKTLGTRLEAKYLCS	35
EBI6	MASSS-GSSP-----RP-----APDEN-----EYFPGCP	23
TRAF1	MASSS-----APDEN-----EYFPGCP	17
LAP1	K--CHLVLCSPKQTECGHRCESCMAALLSSSSPK-CTACO-----ESIVKD	98
TRAF2	A--CKNILARRPFOACCGHRYCSFCLTSILSSGPON-CAACVYEGLYEEGISILES	87
EBI6	PTVCQD--PKEPRALC-----CAGCLSENFRNGEDQICPKRGEDL-----QSISPG	68
TRAF1	PAPCQD--PSEPRVLC-----CTACLSNLRDDIEDRICPKRADNL-----HPVSPG	52
LAP1	-KVFKNCKKREILALQIYCRNESRGCA---EQLMLGHLLVHLKNDCHFEELPCV	149
TRAF2	SSAFFPDNAARREVESLPAVCP--NDGCTWKGT---LKEYESCHEGLCPFLITEC-	136
EBI6	SRLRTQEKARPEVAEAGIGCFFAGVGCSPKGSFQSVQEHVTSQTSNLLLL---	120
TRAF1	SPL-TQEKVSDVAEAEIMCFFAGVGCSPKGSFQSMQEHVTSQSSHYLLL---	113
LAP1	RPDCKEKVLRKDLRDHVEKACKYREATCSHCKSQVPMIALQKHEDTDCPCVVWSC	204
TRAF2	-PACKGLVRLSEKENHTEQECPKRSLSCQHCRAPCSHVQLEVHYEV-CPKFLPTC	189
EBI6	-----GFMKQWKLRLCGGLESQPMALQNL-----SDLQQAAYEV-AGDLEVDC	164
TRAF1	-----AVLKWKSSPGSNLGSAPMALERNL-----SELQQAAYEA-TGDLEVDC	157
LAP1	PHKCSVQTLRLSELSAHLSECYNAPSTCSFKRYGCVFOGTNOQIKAEASSAVOH	259
TRAF2	-DGGCKKKIPRETFOHVRACSKCRVLCRFHTVGC-----SEMVETEN---LQD	234
EBI6	-----YRACP-----SES---QEE	175
TRAF1	-----YRACP-----CES---QEE	168
LAP1	VNLLKENSNSLEKKVSLLONESVEKNKSLHNO--SFEIEDEROKEMRNES	314
TRAF2	HEL-----QRLREHLALLSSFLEAQASPGTLNQVGPPELLORCOILEOKIATFEN	284
EBI6	LAL-----QHFME-----KLAE-----LEGRLVTFEN	199
TRAF1	LAL-----QHLVKE-----KLAE-----LEERLVTFAN	192
LAP1	KHLHLQVQDSQAEKCELDKEPRPFQNWEEADSMKSSDESLOQNVTELESVDK	169
TRAF2	IMCVLNREVERVAVT--AEACSRQHLQDQK-----YEALSNKVQOLE	325
EBI6	IMAVLNKEVEASHLA---LATSISQOLDREH-----LSLEQNVVELO---	240
TRAF1	IMAVLNKEVEASHLA---LAASISQOLDREH-----LSLEQNVVELO---	233
LAP1	SAGQVARNGLLESQSRHDOMSVHDIRADMOLRFQVLETASYNGVLIWKIRD	424
TRAF2	-----RSUGLKDLAMADLEQKUSELEVSTYDGVFIWKISD	350
EBI6	-----OTLQNDQALRLLEOSRLMKEASFQGTFLWKITN	275
TRAF1	-----OTLQNDQALRLLEHSLRLMKEASFQGTFLWKITN	268
LAP1	YKRRKQEAVMGKTLISLYSQPFYTGFGYKMCARVYLNGDGMKGTHLSLFFVIMR	479
TRAF2	FTRKRQEA VAGRTPAIFSPAFYTSRYGYKMLRVYLNGDGTGRGTHLSLFFVVMK	415
EBI6	VTRCHESACGRVSLFSPAFYTTAKYGYKCLRLYLGDDGTGRTHLSLFFVIMR	330
TRAF1	VTRCHESVCGRTVSLFSPAFYTTAKYGYKCLRLYLGDDGSGRTHLSLFFVIMR	323
LAP1	GEYDALLPWPFFKQKVTLMMLDOGSSRRHLGDAFKDPNSSSFKKPTGEMNIASGC	534
TRAF2	GPNDALLQWFFNQKVTLMMLDH--NNREHVIDAFRPDVTSSSFQRPVSDMNIASGC	459
EBI6	GEYDALLPWPFFKQKVTLMMLDQ--NNREHVIDAFRPDLSSASFQRPQSETHVASGC	384
TRAF1	GEYDALLPWPFFKQKVTLMMLDQ--NNREHVIDAFRPDLSSASFQRPQSETHVASGC	377
LAP1	PVFVAQTTLENG--TYIKDDTIFIKVIVDTSDLDPD	568
TRAF2	PLFCPVSKMEA-KNSYVRDDAIFKAIV---DLTGL	501
EBI6	PLFFPLSKLQSPKRAYVKDDTMYLKIV---E-TST	416
TRAF1	PLFFPLSKLQSPKRAYVKDDTMYLKIV---D-TSA	409

Figure 1

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Fig.2

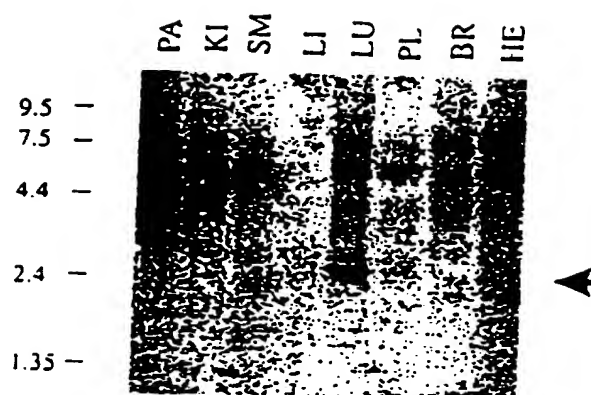
A



LAP1

Fig.2

B



EBI6

Fig.2

C

BL41  
BL41/B95-8  
IB4



LAP1



ACTIN

Fig.2

D

BL41  
BL41/B95-8  
IB4



EBI6



ACTIN

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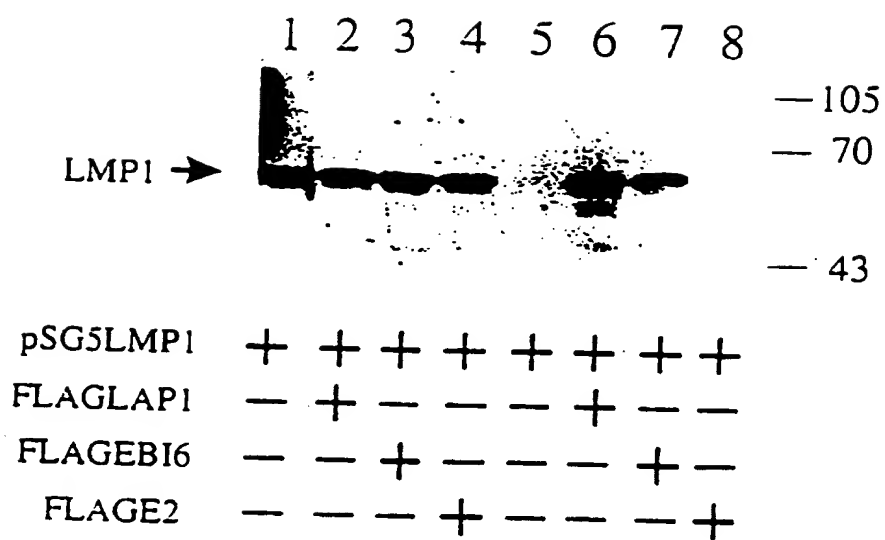


Figure 3



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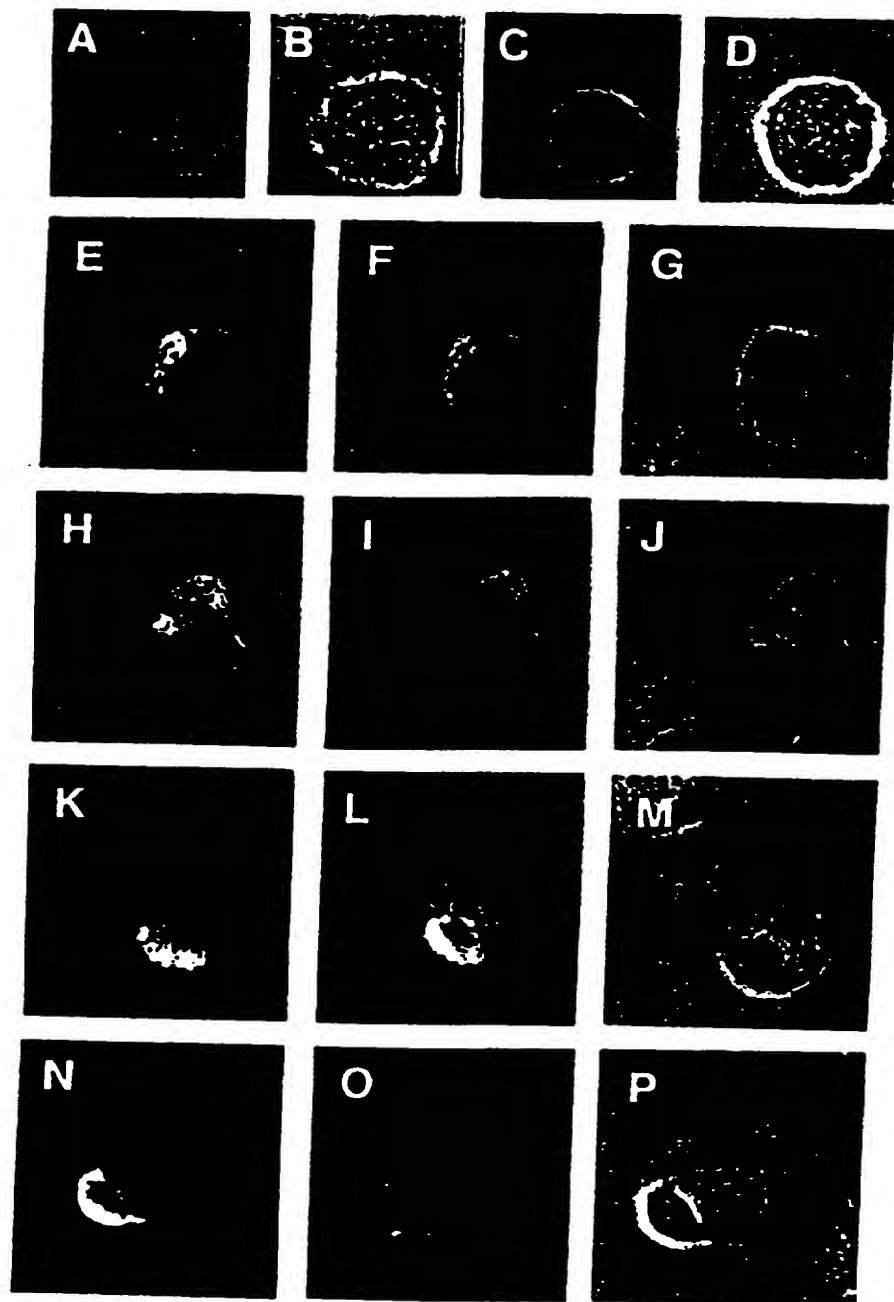


Figure 4

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Fig.5

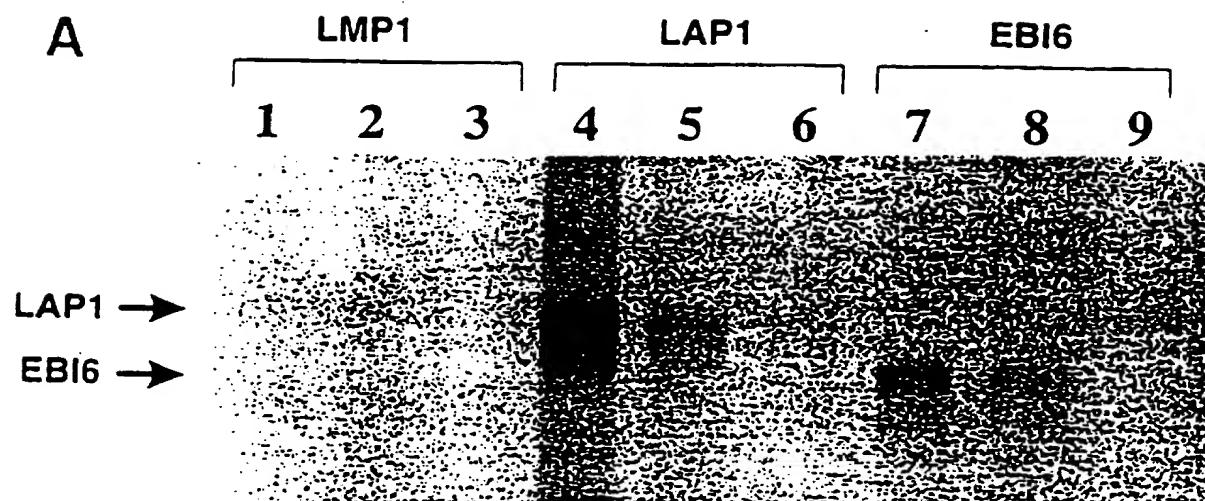
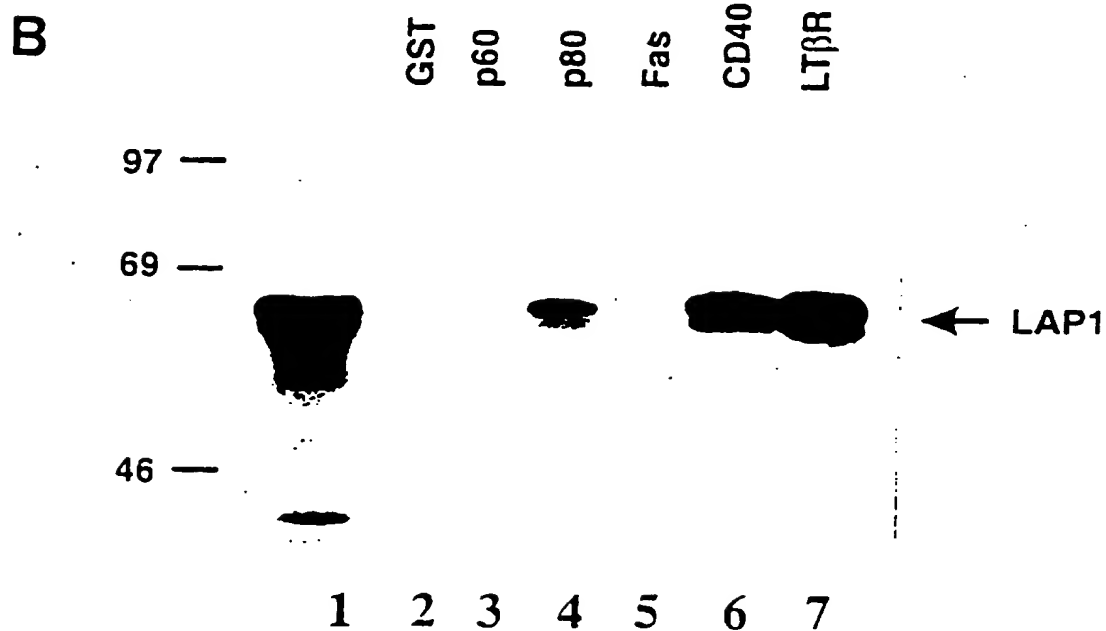


Fig.5



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Fig.5

C



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Fig.6

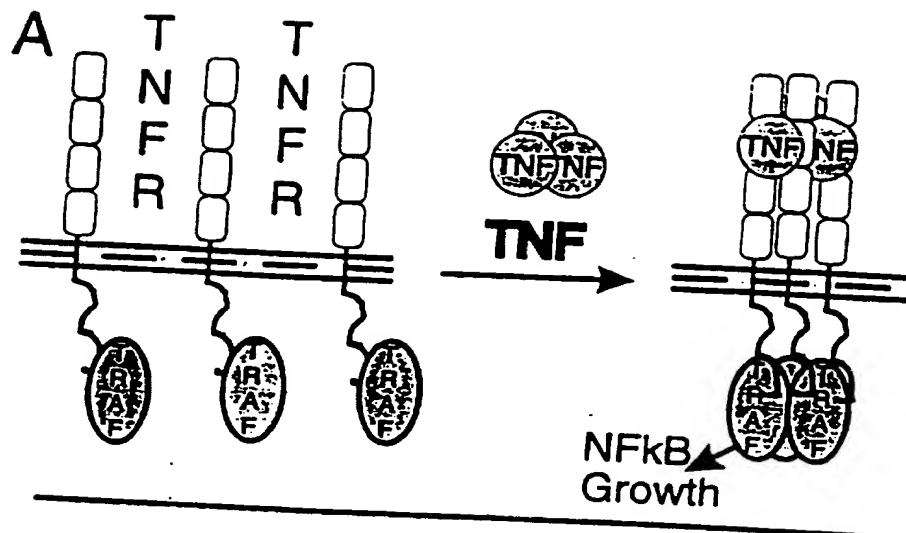
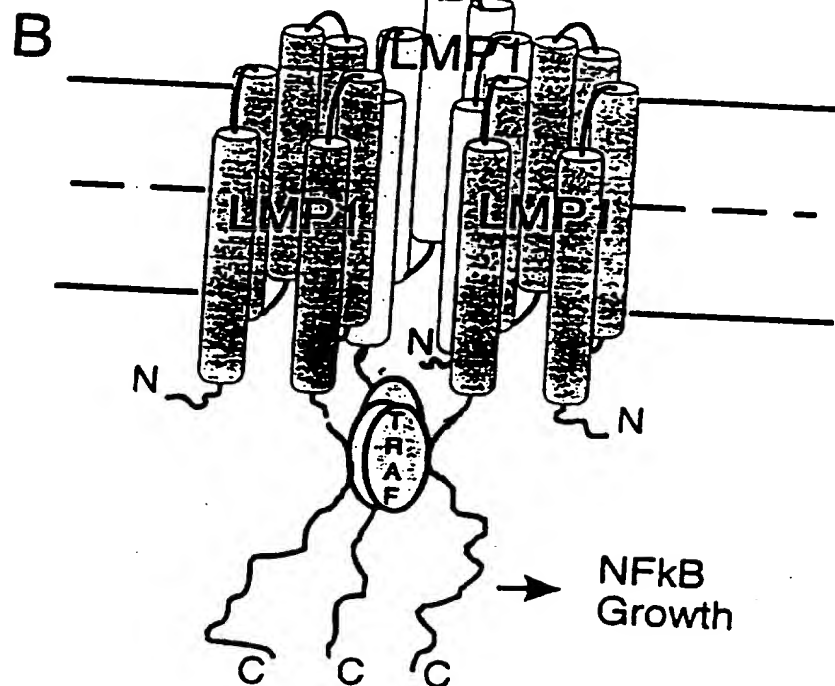


Fig.6



# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/16980

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 38/02; C07K 4/12; C12P 21/02

US CL : 514/12; 530/300, 324, 350; 536/23.5; 435/69.1, 172.1, 252.3

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/12; 530/300, 324, 350; 536/23.5; 435/69.1, 172.1, 252.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Searched Sequences SEQ ID NO: 1-3 on CAS ONLINE

Searched APS, MEDLINE, DIALOG

search terms: TRAF, TNFR, EBV, LMP1, LAP1, method of treatment

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X ----- P, Y	Cell, Volume 80, issued 10 February 1995, Mosialos et al, "The Epstein-Barr Virus Transforming Protein LMP1 Engages Signalling Proteins for the Tumor Necrosis Factor Receptor Family", pages 389-399, especially pages 390 and 392-395.	72-74 ----- 1-51 and 53
X, P ----- Y, P	Journal of Virology, Volume 69, No. 2, issued February 1995, Kaye et al, "The Epstein-Barr Virus LPM1 Cytoplasmic Carboxy Terminus Is Essential for B-Lymphocyte Transformation; Fibroblast Cocultivation Complements a Critical Function within the Terminal 155 Residues", pages 675-683, especially pages 676 and 681-682.	72-74 ----- 1-51 and 53

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be part of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*&*	document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means		
*P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

23 APRIL 1996

Date of mailing of the international search report

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**BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING**

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s) 1-51, 53 and 72-74 drawn to a medicament for treating Epstein-Barr Virus (EBV)-associated infection, cell growth, tumorigenesis, rheumatoid arthritis, Crohn's disease or lupus by administering a polypeptide compound that inhibits TRAF/TNFR cell growth/death signal transduction; the process of making the medicament and the process of using the medicament.

Group II, claim(s) 52, drawn to a medicament for controlling TRAF-Mediated TNF/TNFR signal transduction, said medicament comprising purified nucleic acid or a cell expressing purified nucleic acid.

Group III, claim(s) 54-62, drawn to a method for in vitro screening for substances that inhibit Tumor necrosis factor Receptor Associated Factor (TRAF) protein related signal transduction.

Group IV, claim(s) 63-64, drawn to a method for in vivo screening for substances that inhibit the interaction of LMP1 with a Tumor necrosis factor Receptor Associated Factor (TRAF) protein.

Group V, claim(s) 65-69, drawn to a method for in vitro screening for substances that inhibit the interaction of Tumor necrosis factor Receptor Associated (TRAF) protein with a member of the tumor necrosis factor receptor family.

Group VI, claim(s) 70-71, drawn to antibodies that specifically bind to a LAP1 or EB16 immunodeterminant.

The inventions listed as Groups I-VI do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Group I recites the special technical feature of administering a polypeptide compound that inhibits TRAF/TNFR cell growth/death signal transduction that is not needed for the inventions of Groups II-VI.

Group II recites the special technical feature of administering purified nucleic acid or a cell expressing purified nucleic acid for controlling TRAF-Mediated TNF/TNRF signal transduction that is not needed for the inventions of Groups I and III-VI.

Group III recites the special technical feature of an in vitro screening method for substances that inhibit Tumor necrosis factor Receptor Associated Factor (TRAF) protein related signal transduction that is not needed for the inventions of Groups I-II and IV-VI.

Group IV recites the special technical feature of an in vivo screening method for substances that inhibit the interaction of LMP1 with a Tumor necrosis factor Receptor Associated Factor (TRAF) protein that is not needed for the inventions of Groups I-III and V-VI.

Group V recites the special technical feature of an in vitro screening method for substances that inhibit the interaction of Tumor necrosis factor Receptor Associated (TRAF) protein with a member of the tumor necrosis factor receptor family that is not needed for the inventions of Groups I-IV and VI.

Group VI recites the special technical feature of antibodies that specifically bind to a LAP1 or EB16 immunodeterminant that are not needed for the inventions of Groups I-V.